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Neuraminidases as triggers of atherosclerosis

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Dedicated to

My family, Guillaume and Maxime

Summary

Atherosclerosis is a chronic vascular disease characterized by lipid retention and inflammation in the vessel wall. Atherosclerosis of the coronary arteries (Coronary Artery Disease or CAD) is the leading current cause of death worldwide. The disease is triggered by the uptake by resident macrophages of low-density lipoproteins (LDL) forming arterial fatty streaks and eventually atheromatous plaques. Our recent data suggest that enzymes of the neuraminidase (sialidase) family, present on the surface of hematopoietic cells and arterial endothelium, also contribute to atherosclerosis. These enzymes are involved in removal of sialic acids (Sia) from glycan chains of glycoproteins and glycolipids, modulating molecular and cellular recognition as well as cellular signaling pathways. Previously human neuraminidases (Neu1, Neu2, Neu3, and Neu4) were not considered to be clinical targets for prevention or treatment of atherosclerosis, but we found that desialylation of a major LDL glycoprotein – apolipoprotein B 100 (ApoB) in human LDL by Neu1 or Neu3 increases LDL uptake by cultured human macrophages and leads to accumulation of LDL in the aortic wall of live mice. We further showed that in a murine model of atherosclerosis, Apolipoprotein E (*ApoE)* knockout mice, genetic deficiency of Neu1 and Neu3 significantly delayed the formation of fatty streaks in the aortic root. We suggest that neuraminidase inhibition-based therapies merit clinical evaluation for treating or preventing atherosclerosis and coronary artery disease.

Key words: atherosclerosis, coronary artery disease, neuraminidase, sialic acid, low-density lipoproteins, apolipoprotein B 100.

Résumé

L'athérosclérose est une maladie vasculaire chronique qui se caractérise par une accumulation de lipides et une inflammation dans la paroi artérielle. L'athérosclérose est un facteur de risque majeur de la maladie artérielle coronarienne qui représente actuellement la principale cause de mortalité dans le monde. La maladie se déclenche lorsque les macrophages résidents captent les lipoprotéines de basse densité (LDL) formant ainsi la strie lipidique et éventuellement une plaque d'athérome. Nos récentes données suggèrent que les enzymes de la famille des neuraminidases (sialidases), présentes à la surface des cellules hématopoïétiques et de l'endothélium vasculaire, contribuent au développement de l'athérosclérose. Les neuraminidases catalysent la suppression des résidus d'acides sialiques (Sia) présents dans les chaînes glycanes des glycoprotéines et des glycolipides, modulant ainsi les événements de reconnaissance moléculaire et cellulaire ainsi que les voies de signalisation. Jusqu'alors les neuraminidases humaines (Neu1, Neu2, Neu3, et Neu4) n'ont pas été considérées comme des cibles thérapeutiques potentielles pour la prévention ou le traitement de l'athérosclérose. Nous avons démontré que la desialylation par Neu1 et Neu3 de l'apolipoprotéine B 100 (ApoB), glycoprotéine majeure contenue dans les LDL humains, augmente la captation des LDL par les monocytes humains en culture ainsi que l'accumulation des LDL dans la paroi artérielle de souris sauvages. De plus, dans un modèle murin d'athérosclérose, les souris déficientes en apolipoprotéine E (*ApoE* KO), la déficience génétique en Neu1 et Neu3 diminue significativement la formation de la strie lipidique au niveau du sinus aortique. L'ensemble de ces résultats suggèrent que des thérapies basées sur l'inhibition des neuraminidases méritent une évaluation

clinique pour la prévention et le traitement de l'athérosclérose et des maladies artérielles coronariennes.

Mots clés : athérosclérose, maladie artérielle coronariennes, neuraminidases, acide sialique, lipoprotéines de basse densité, apolipoprotéine B 100.

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Abbreviations

4-Mu-NANA	4-Methylumbelliferyl-N-acetyl-α-D-neuraminic acid
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ACAT	Acyl-CoA cholesterol acyltransferase
AKT	Protein kinase B
Alexa	Fluorescent dye
ApoB	Apolipoprotein B
ApoC	Apolipoprotein C
ApoE	Apolipoprotein E
Arg	Arginine
Asn	Asparagine
ATP	Adenosine-5'-triphosphate
BODIPY	Boron-dipyrromethene
BSA	Bovine serum albumin
CAD	Coronary artery disease
CathA	Cathepsin A
CD	Cluster of differentiation
CETP	Cholesterol ester transfer protein
CM	Chylomicron
COS 7	Fibroblast-like cell line derived from monkey kidney tissue
DANA	2-deoxy-2,3-dehydro-N-acetylneuraminic acid
DAPI	4, 6-diamidino-2-phenylindole
desLDL	Desialylated low-density lipoprotein
DiI	3,3'-dioctadecylindocarbocyanine
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence substrate
EDTA	Ethylenediamine tetraacetic acid

ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FcγR	Fc-gamma receptors
FFA	Free fatty acid
FPLC	Fast protein liquid chromatography
Fuc	Fucose
Gal	Galactose
GalNAc	N-Acetylgalactosamine
Glc	Glucose
GlcNAc	N-acetyl-D-glucosamine
G_M	Monosialotetrahexosyl ganglioside
GST	glutathione S-transferase fusion proteins
HDL	High density lipoprotein
HEK293	Human embryonic kidney cells 293
HepG2	Human liver cancer cell line
HDL	High density lipoprotein
His	Histidine
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HRP	Horseradish peroxidase
HTGL	Hepatic triglyceride lipase
ICAM-1	Intercellular adhesion molecule-1
IDL	Intermediate-density lipoprotein
IL	Interleukin
INF- γ	Interferon-γ
KI	knockin
K_M	Michaelis constant
KO	knockout
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein

LDLR	Low-density lipoprotein receptor
LOX-1	Lectin-like oxLDL receptor
Lp(a)	lipoprotein(a)
LPDS	Lipoprotein deficient serum
LPL	Lipoprotein lipase
LRP	Low-density lipoprotein receptor-related protein
MAL	Maackia amurensis leukoagglutinin
MBP	Maltose-binding protein
MHC II	Major histocompatibility complex-2
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage-colony stimulating factor
mRNA	Messenger RNA
MS/MS	Tandem mass spectrometry
MTTP	Microsomal triglyceride-transfer protein
Neu1	Neuraminidase 1
Neu2	Neuraminidase 2
Neu3	Neuraminidase 3
Neu4	Neuraminidase 4
Neu5Ac	N-Acetylneuraminic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nLDL	Native low-density lipoproteins
NPT	Isothermal–isobaric ensemble
OCT	Optimum cutting temperature compound
oxLDL	Oxidized LDL
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
PME	Particle mesh Ewald

PMN	Polymorphonuclear leukocyte
PNA	Peanut (Arachis hypogaea) agglutinin
Rho	Kinase belonging to the AGC family of serine-threonine kinases
RMPI	Roswell Park Memorial Institute medium
RNA	Ribonucleic acid
S	Substrate
SDS	Sodium dodecyl sulphate
Sia	Sialic acid
SMC	Smooth muscle cell
SNA	Sambucus nigra agglutinin
SR-A	Scavenger receptor A
SR-B1	Scavenger receptor class B member 1
SREBP	Sterol regulatory element-binding protein
TBS	Tris buffered saline
TG	Triglyceride (triacylglycerol)
THP-1	Tamm-Horsfall protein 1 (human acute monocytic leukemia cell line)
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein
V_{max}	Maximal velocity of enzymatic hydrolysis
WT	Wild type

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Chapter 1. General introduction

1.1 Structure of arterial wall

The arterial wall is composed of three morphologically distinct layers that surround the luminal cavity (Figure 1). The innermost layer – the tunica intima in its turn has three layers: the endothelium, the intima and the basement membrane (Lusis, 2000). An endothelium monolayer acts as a physical and functional barrier from the blood stream; besides endothelial cells play a regulatory role in the arterial biology participating in blood pressure regulation, leukocyte trafficking and vascular tone regulation via the production of vasoactive mediators (Sudano et al., 2006). The outer membrane of the tunica intima known as the elastica interna separates it from the next layer – the tunica media. The tunica media consists of concentric layers of vascular smooth muscle cells. The next layer, the elastica externa separates the tunica media from the outermost layer of the arterial wall, the tunica adventitia, which consist of connective tissue with sporadic fibroblasts, progenitor cells and muscle cells (Lusis, 2000).

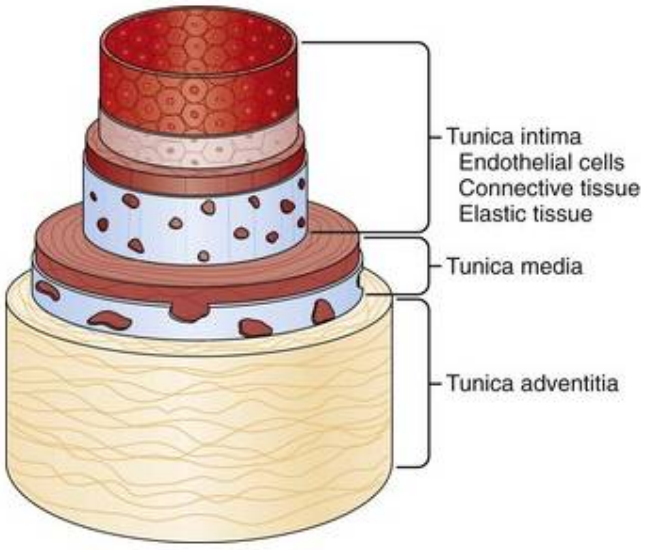

Figure 1. Structure of the arterial wall.
(Hast, 2003)

1.2 Atherosclerosis overview

The term atherosclerosis has a Greek origin, and means thickening of the intimal layer of arteria and accumulation of fat (*athere* meaning gruel, and *skleros* meaning hardness) (Mallika, Goswami, & Rajappa, 2007). Atherosclerosis is a progressive inflammatory disease characterized by accumulation of lipids and fibrous elements in arteries of all sizes with the most dramatic effects in the aorta, coronary and cerebral arteries (Figure 2, 3). Atherosclerosis leads to coronary artery disease (CAD), one of the leading causes of mortality in western society (Libby, 2002). According to a World Health Organization report published in 2016 as a part of "Global Hearts" initiative cardiovascular disease is responsible for over 17.5 million deaths annually (~31% of all deaths) and this value is projected to increase to 23.6 million annual deaths by 2030 (World Health Organization, 2017). The disease is initiated by infiltration of the subendothelial space of the artery wall by cholesterol-carrying low-density lipoprotein (LDL) particles from the circulation (Lusis, 2000). LDL particles become modified and recognized by residential macrophages, which leads to uncontrolled accumulation of cholesterol in these cells. The appearance of lipid loaded macrophages – foam cells – is the main characteristics of the early stage of atherosclerosis (X. H. Yu, Fu, Zhang, Yin, & Tang, 2013). This is a complex process, that involves a large number of growth factors, cytokines, regulatory molecules and different cell types (Koenen & Weber, 2010). Activation of endothelial cells of the arterial wall leads to secretion of proinflammatory cytokines and chemokines and increased expression of the adhesion molecules on their surface (Steinberg, 1997). It results in recruitment and attachment of circulating monocytes that migrate into the

arterial wall and become macrophages and eventually, foam cells. Further, foam cells combine to form arterial fatty streaks.

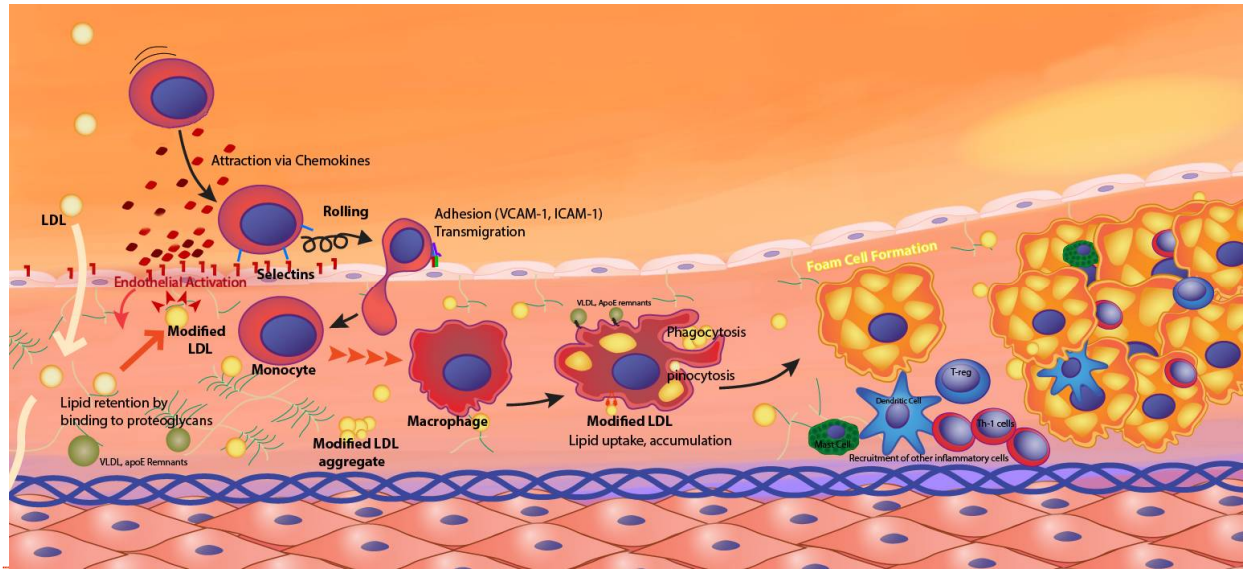

Figure 2. Initiation of atherosclerosis: formation of the fatty streak.
The fatty streak phase of atherosclerosis begins with dysfunctional endothelial cells and the retention of LDL in the subendothelial space. Retained LDL become modified (oxidation, glycation, enzymatic processing) and promote activation of endothelial cells. Activation of endothelial cells results in monocytes migration into the intimal space. The monocytes differentiate into macrophages and express receptors that mediate the internalization of modified LDL to become foam cells. Inflammatory signaling pathways are activated in macrophage foam cells leading to more cell recruitment and LDL modification. Modified from Linton et al. (Linton et al., 2000).

In humans, fatty streak lesions can be found in the aorta already during the first decade of life. In general, they do not have any clinical significance, but may become precursors of more advanced lesions. By the third decade of life they can occupy as much as one third of the aorta surface (Stary et al., 1995). These fatty deposits called atheromatous plaques appear in the inner layers of arteries in certain areas, that correspond to the regions of branching or high vessel curvature (Davies, 2000). These areas have specific hemodynamic features and are typically associated with low shear stress and oscillatory or turbulent flow (Zarins et al., 1983). These factors promote the appearance of a proinflammatory endothelial cell phenotype, that leads to

production of leukocytes adhesion molecules, increased NADPH oxidase activity and other events contributing to chronic inflammation (Cybulsky & Jongstra-Bilen, 2010; Libby, Ridker, & Maseri, 2002).

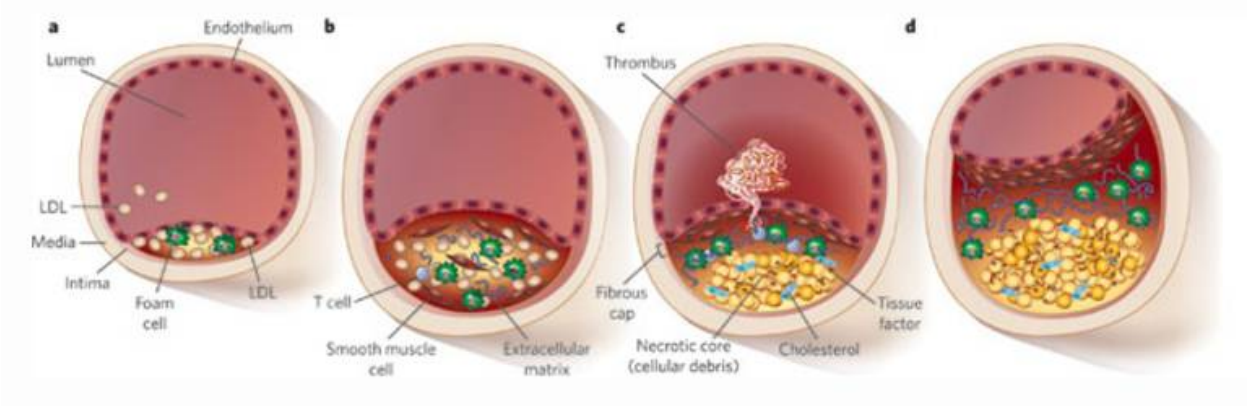

Figure 3. Initiation and progression of atherosclerosis.
a, Atherogenic LDL enter the intima. Unrestricted uptake of atherogenic lipoproteins by macrophages leads to the generation of foam cells. The accumulation of foam cells results in the formation of fatty streaks. **b**, SMC secrete large amounts of collagen. **c**, Death of foam cells causes release of cellular debris and crystalline cholesterol. SMC form a fibrous cap beneath the endothelium that separates plaque from the blood stream. The plaque can rupture or the endothelium can erode, resulting in the formation of a thrombus. The thrombus can block the artery, which causes an acute coronary syndrome or myocardial infarction (heart attack). **d**, If the plaque does not rupture and the lesion continues to grow, the lesion can encroach on the lumen and result in clinically obstructive disease (Rader & Daugherty, 2008).

At some point healthy macrophages become unable to keep up with necrosis and apoptosis of foam cells and this stage corresponds to the development of the necrotic lipid core (Tabas, 2010). The deposition of cholesterol crystals in the arterial wall and its underlying smooth muscle cell (SMC) leads to the formation of atherosclerotic plaques. The SMC proliferate and migrate into advanced lesions. SMC also produce cytokines and growth factors (IL-1, TNF), which cause migration of SMC into the luminal side of the arterial wall (Steinbrecher, Parthasarathy, Leake, Witztum, & Steinberg, 1984). Proteinases secreted by activated leukocytes damage the extracellular matrix and at the same time pro-inflammatory cytokines downregulate synthesis of

new collagen. Collagen covers the surface of the plaque, forming a fibrous cap that protects the necrotic core and stabilizes the lesion. (Hopkins, 2013). The growing lipid core thins the fibrous cap and makes it susceptible to rupture. Once the plaque ruptures, platelets activated by thrombin initiate thrombus formation. A thrombus can result in complete blockage of blood flow causing a heart attack or stroke (Libby, Ridker, Hansson, & Leducq Transatlantic Network on, 2009; Ross, 1993).

1.3 Risk factors of atherosclerosis

The exact causes of atherogenesis are still unclear, but atherosclerotic lesions can occur throughout life. The earliest type of lesions, the fatty streaks are common in young children and even in infants (Napoli et al., 1997). They are qualified as strictly inflammatory lesions because they consist only of macrophages and T-lymphocytes (Stary et al., 1994). Although all risk factors of atherogenesis are still unknown, certain conditions and habits have been shown to increase the chance of developing the disease. First of all, there is a strong correlation between total serum cholesterol and increased risk of atherosclerosis (Law, Wald, & Thompson, 1994). Also, a number of non-lipoprotein risk factors have been identified. They include age, sex, genetic predisposition, tobacco smoking, hypertension, type II diabetes, obesity and the lack of physical activity (Tegos, Kalodiki, Sabetai, & Nicolaides, 2001). It has been shown that women have a lower risk than men, because of the antiatherogenic effects of estrogen hormones during the premenopausal period (Kalin & Zumoff, 1990).

The association of defects in lipid metabolism with the risk of CAD has been examined for decades. Major studied parameters were the total blood cholesterol, triglycerides and

lipoprotein cholesterols including low density lipoprotein (LDL) cholesterol and high density lipoprotein (HDL) cholesterol (Grundy, 1995). These studies identified a conventional rule for the atherosclerosis risk: the less LDL, the better (Carmena, Duriez, & Fruchart, 2004), which is opposite for HDL – the more the better (Superko et al., 2012).

1.4 Inflammation in atherosclerosis

Inflammation is recognized as a central pathogenic process in atherosclerosis (Libby et al., 2009). However, it is not a single inflammatory cascade that is involved in atherogenesis, instead, it involves all branches of the immune system including innate immunity, adaptive immunity and humoral immunity.

The first steps of the inflammatory process in atherosclerosis belong to the innate immunity – its components such as monocytes, dendritic cells, mast cells and platelets are retaliated since the beginning of the pathogenic process (Libby et al., 2009). Activated endothelial cells of the arterial intima attract circulating monocytes via leukocyte adhesion molecules, such as E-selectin, P-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Rocha & Libby, 2009). It has been shown that endothelial cells express VCAM-1 in response to modified LDL (Nakashima, Raines, Plump, Breslow, & Ross, 1998). Also, chemokines induce migration of the monocytes to the intima. The chemokines best known to be associated with atherosclerosis are monocyte chemoattractant protein-1 (MCP-1) and its receptor, CC-receptor-2, CC-chemokine ligand 5 and its receptor, CC-receptor 5 as well as CX3-chemokine receptor-1 (Boring, Gosling, Cleary, & Charo, 1998). The activation of macrophages in the intima leads to upregulation of scavenger and toll-like receptors (TLR) that recognize a large

spectrum of molecules, including modified LDL and apoptotic cell fragments (Janeway & Medzhitov, 2002). Stimulation of macrophages through TLR and scavenger receptors leads to activation of the proinflammatory transcription factor – nuclear factor κ-B (NF-κB) and subsequent expression of various cytokines (Vatten & Kvinnsland, 1990). With the appearance of lipid-rich foam cells, macrophages release growth factors and cytokines, which induce further inflammation. They also secrete proteases that degrade collagen and stimulate apoptosis of smooth muscle cells (Hansson, 2005).

The adaptive immune response is involved in atherogenesis due to the requirement of T-lymphocytes, since they are as essential to atherosclerotic plaque as monocytes (Mallat, Taleb, Ait-Oufella, & Tedgui, 2009). CD4+ T-cells or helper T-cells are the major lymphocyte subclass in the atheromatous plaque. Th1 cells – the subtype of helper T-cells, are known to induce production of proinflammatory cytokines, such as interferon-γ (INF- γ) and tumor necrosis factor-α (TNF-α). In its turn, expression of these cytokines upregulates production of many other inflammatory and cytotoxic factors in macrophages and vascular cells, that leads to further development of atherosclerosis (Hansson, 2005). 30% of T-cells in human atheromatous plaques are CD8+ T-cells, which are capable of destroying other cells via cell-cell contact (Libby et al., 2009). Activation of these cells in mice leads to destruction of smooth muscle cells and macrophages in arteries, which accelerates atherosclerosis (Ludewig et al., 2000). Lipid antigens activate natural killer T-cells that are also present in early atherosclerotic lesions (Tupin et al., 2004). In contrast, B-cells responsible for humoral immunity have anti-atherogenic effects (Caligiuri, Nicoletti, Poirier, & Hansson, 2002).

1.5 Recognized mechanisms for triggering atherogenesis

Since clinical observations showed that development of atherosclerosis has a non-random pattern, multiple studies have been focused on the investigation of the mechanism triggering this disease (Ross, 1993; Schwartz, Valente, Sprague, Kelley, & Nerem, 1991; Stary et al., 1994). Various hypotheses have been proposed to explain monocyte recruitment and lipid loading of the differentiated macrophages. The three most recognized (elaborated) hypotheses of atherogenesis are: "response to injury", "response to retention" and "oxidative modification".

Response to injury -

This hypothesis proposes that atherogenesis is initiated by injury and denudation of the endothelial cell monolayer of the intima, leading to monocyte and platelet recruitment to the site of the injury and induction of the inflammatory response. However, the fact that an intact endothelium can also give rise to the progression of atherosclerosis weakens the idea of injury being the initiating factor of the disease and prompts alternative hypotheses for the initiation of atherosclerosis (Ross, 1999).

Response to retention -

According to this hypothesis the central pathogenic process in atherogenesis is subendothelial LDL retention (Nievelstein, Fogelman, Mottino, & Frank, 1991). The retention of lipoprotein involves the association of major LDL apolipoprotein, ApoB with proteoglycans in the arterial wall (Camejo, Fager, Rosengren, Hurt-Camejo, & Bondjers, 1993; Yla-Herttuala et al., 1987). This theory has been supported by evidence that transgenic mice expressing mutant ApoB incapable of binding to proteoglycans have a greatly reduced atherogenic potential (Flood et al.,

2002; Skalen et al., 2002; Veniant et al., 1997). Another argument that supports the "response to retention" hypothesis is the presence of lipolytic and lysosomal enzymes in the extracellular matrix, since binding of LDL to endothelial cells is strongly dependent on the activity of lipoprotein lipase (the enzyme that hydrolyzes lipoprotein triglycerides into free fatty acids) (Williams, Petrie, Brocia, & Swenson, 1991). Lysosomal enzymes including cathepsin D and lysosomal acid lipase (Hakala et al., 2003) also induce aggregation of LDL and proteoglycans and avidly facilitate foam cell formation in the arterial wall (Ismail, Alavi, & Moore, 1994).

Oxidative modification -

Oxidative modification is the most well-known hypothesis of atherogenesis mechanism. It focuses on the concept that LDLs themselves are not atherogenic until they become modified. LDL trapped in the sub-endothelial region can undergo oxidation mediated by the endothelial cells, macrophages and smooth muscle cells (Stocker & Keaney, 2004). Oxidized LDL (oxLDL) becomes then recognized by macrophages through the scavenger receptor pathway resulting in uncontrolled uptake of LDL and formation of foam cells (Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989). Importantly, antioxidant supplements such as vitamins E and C alone as well as potent non-vitamin antioxidant (succinobucol) provided no benefit to CAD patients in secondary prevention (Steinberg & Witztum, 2002; Tardif, Gregoire, et al., 2008; Tardif, McMurray, et al., 2008; Thomson, Puntmann, & Kaski, 2007). Besides, multiple studies reviewed below have shown that there are many other modifications of LDL that can be responsible for inducing their accumulation in macrophages. Thus, the theory of oxidative modification can be considered as popular, but not central.

1.6 Treatment and prevention of atherosclerosis

The primary prevention of atherogenesis is based on promoting a healthy lifestyle including normal body weight, increased physical activity, smoking cessation and proper nutrition habits. However, for millions of people at high risk of atherosclerosis complications, lifestyle changes are not enough to prevent the disease. Current clinical treatments of atherosclerosis in general are based on the drugs that lower plasma cholesterol and blood pressure (Chhatriwalla et al., 2009; Ishii et al., 2013; Roy, 2014), yet it does not help to completely eliminate mortality associated with atherosclerosis.

Two thirds of the total cholesterol are synthesized in the body, the rest coming from food. Statins, the most clinically and financially successful drugs for the treatment of atherosclerosis, target the cholesterol de novo synthesis pathway by inhibiting the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Babelova, Sedding, & Brandes, 2013; Ray, Cannon, & Braunwald, 2007). At the same time, all statins raise the level of HDL, which is considered as an antiatherogenic effect (Barter, Brandrup-Wognsen, Palmer, & Nicholls, 2010). Inhibitors of the HMG-CoA reductase also have immunomodulatory effects due to inhibition of interferon gamma (IFN-γ) induced major histocompatibility complex-2 (MHC II)-mediated T-cells activation and expression of monocyte adhesion molecules (Danesh et al., 2003). Statins also induce Akt and Rho/Rho-kinase systems that inhibit migration of smooth muscle cells and mobilize endothelial progenitor cells (Q. Zhou & Liao, 2009). Through this mechanism inhibitors of the HMG-CoA reductase reduce plaque progression even without lowering LDL level. However, the high doses of statins needed for a sufficient lowering LDL-cholesterol were found to have a greater risk of

side effects (Grundy, 2002). In particular, it has been shown that statins inhibit the synthesis of vitamin K_2 and accelerate artery calcification (Saremi, Bahn, Reaven, & Investigators, 2012). Another study showed that statins decrease the concentration of mitochondria in muscle tissues (Larsen et al., 2013) and lead to other adverse effects, including stimulation of atherosclerosis, heart failure, carcinogenicity, central and peripheral nervous disorders and hepatic injury (de Lorgeril & Salen, 2014; Okuyama et al., 2015).

Blocking absorption of cholesterol in the intestine becomes another clinical approach to reduce LDL and total circulating cholesterol. Ezetimible is a first agent which is known to inhibit absorption of cholesterol from the lumen of the intestine (Meng, 2002). The complex of caveolin-1 and annexin-2 is the target of ezetimible in regulating intestinal cholesterol transport (Smart, De Rose, & Farber, 2004). This medication in combination with low doses of statins was as effective in lowering LDL-cholesterol as high doses of statins (Kerzner et al., 2003).

Some compounds such as fibric acid also improve the lipid profile, however they do not show significant results in reducing cardiovascular risk in patients (Zoungas & Patel, 2010). Another potential target for reducing atherogenesis is the prevention of leukocyte adhesion and migration through endothelium (Zeiher, Fisslthaler, Schray-Utz, & Busse, 1995). Several preclinical studies have demonstrated that vitamin E (α-tocopherol), the major lipid-soluble antioxidant of LDL, prevents the progression of atherosclerosis. However, the results of clinical trials are still controversial (Cherubini et al., 2005).

The inhibitors of proprotein convertase subtilisin/kexin type 9 (PCSK9) represent a novel class of anti-cholesterol drugs. Once the LDLR and LDL particle complex undergoes endocytosis, PCSK9 binds to LDLR, marking the receptor for lysosomal degradation (Abifadel et al., 2003;

Verbeek, Stoekenbroek, & Hovingh, 2015). The increased activity of PCSK9 has been associated with high LDL cholesterol and premature CAD (Steinberg & Witztum, 2009). Recently, monoclonal antibody therapies to inhibit PCSK9 were suggested as a potential drug to treat atherosclerosis. In preclinical experiments the antibody showed significant reduction of LDL cholesterol encouraging further development (Schmidli, 2016). A monoclonal antibody to PCSK9, Bococizumab, was being developed by Pfizer, however the company withdrew the drug from development in November 2016, explaining that it was "not likely to provide value to patients, physicians or shareholders" (Danehy, 2016). Other PCSK9 inhibitors, alirocumab (Praluent; Sanofi/ Regeneron, Bridgewater, NJ, USA) and evolocumab (Repatha; Amgen, Thousand Oaks, CA, UDA), showed significant effectiveness in lowering LDL cholesterol in patients with familial hypercholesterolemia in monotherapy or taken together with statins (AlHajri, AlHadhrami, AlMheiri, AlMutawa, & AlHashimi, 2017; Colletti, Derosa, & Cicero, 2016; Lepor & Kereiakes, 2015), however their long-term efficacy in preventing atherosclerosis has yet to be evaluated.

1.7 Mouse models of atherosclerosis

Wild type mice are resistant to atherosclerosis due to a favorable lipoprotein profile (Meir & Leitersdorf, 2004): most of the cholesterol in mouse circulation is carried by anti-atherogenic HDL (Jawien, Nastalek, & Korbut, 2004). However, being fed a diet containing high fat and/or high cholesterol *C57bl/6J* mice develop small atherosclerotic lesions in the aortic root (Paigen, Morrow, Brandon, Mitchell, & Holmes, 1985). The standard laboratory diet for mice contains approximately 6% of fat by weight and a minor amount of cholesterol. Several formulations of diets based on elevated levels of fat and cholesterol were developed to induce or accelerate

atherosclerosis in mice (Getz & Reardon, 2006). The most commonly used atherogenic diet is a so-called Western type diet that is usually comprised of 21% fat and 0.15% cholesterol by weight (Plump et al., 1992).

Genetic inactivation of proteins involved in LDL and VLDL clearance in the *C57bl/6J* mice resulted in reliable murine models of experimental atherosclerosis (Daugherty, 2002). The two most used are *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mice. In the case of A*poE*$^{-/-}$ mice atherosclerosis develops already on the normal diet, but it can be accelerated if mice are fed the Western diet. Inducing atherosclerosis in *Ldlr*$^{-/-}$ mice requires a high fat diet. These mice develop atherosclerosis plaques mainly in the areas of large arteries where blood flow is non-laminar. First atherosclerotic plaques in both models appear in the aortic sinus, the dilation of the aorta that is represented by a three-leaflet valve structure and separates the left heart ventricle from the ascending aorta. The next regions where lesions appear are the aortic arch, brachiocephalic artery and descending aorta (Reardon & Getz, 2001).

1.7.1 ApoE$^{-/-}$ mouse model of atherosclerosis

The important role in chylomicron and VLDL metabolism belongs to apolipoprotein E (ApoE) that is synthesized by the liver cells and is instrumental for the clearance of chylomicrons and VLDL remnant lipoprotein particles via LDLR and LRP (Meir & Leitersdorf, 2004) (discussed in details further in the chapter 1.12.1). Mice deficient in ApoE have a lipid profile similar to that of humans with the majority of cholesterol present as chylomicrons, VLDL, and LDL rather than HDL. They also have a high total plasma cholesterol as compared to that of wild type mice (~10-15 mM vs. ~2 mM) even when they are maintained on a regular low fat, low cholesterol diet (Plump et

al., 1992; S. H. Zhang, Reddick, Piedrahita, & Maeda, 1992). *ApoE*$^{-/-}$ mice fed on a regular diet develop spontaneous atherosclerotic lesions resembling human plaques at a relatively young age (3-5 month) (Nakashima, Plump, Raines, Breslow, & Ross, 1994). Early lesions such as adhesion of monocytes to the aortic wall in the aortic sinus occur at approximately 2 months of age, progressing to formation of foam cells-rich fatty streaks by 3 months. The intermediate lesions can be observed by 15 weeks, fibrous plaques by 20 weeks, calcification and wall thinning by 32 weeks of age (Meir & Leitersdorf, 2004). As in humans, atherogenesis in *ApoE*$^{-/-}$ mice can be further accelerated by keeping them on high fat diet (Figure 4) (Jawien et al., 2004). For all these reasons *ApoE*$^{-/-}$ mice are considered to be the most reliable mouse model of atherosclerosis and are widely used in atherosclerosis studies.

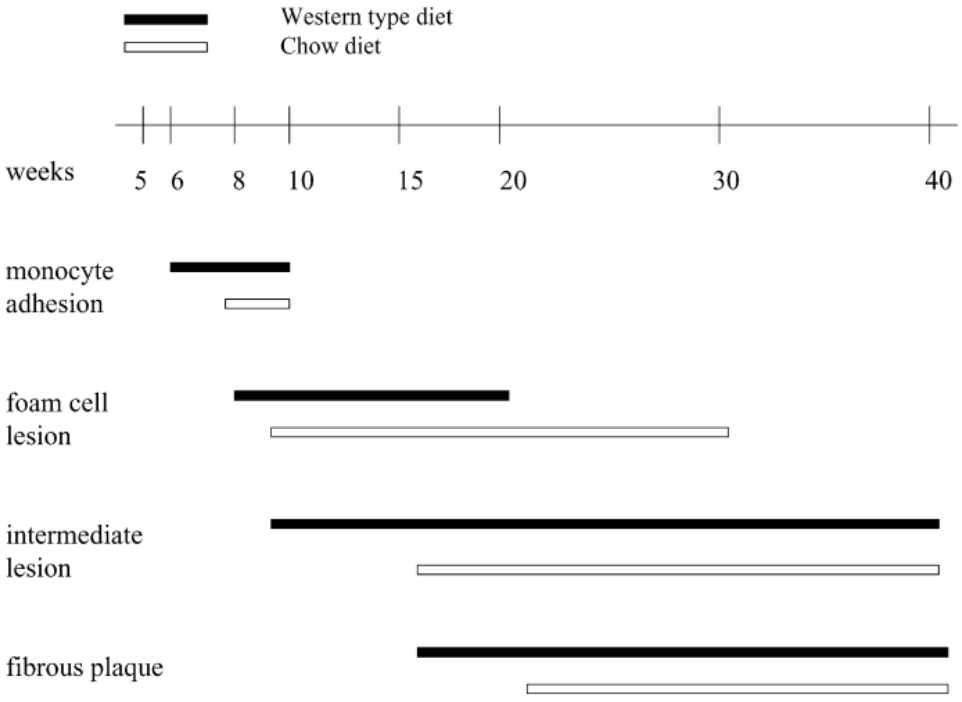

Figure 4. Timeline of lesion formation in the *ApoE*$^{-/-}$ mice.
Diagram illustrates delayed lesion formation in chow-fed mice as compared with mice fed the Western diet (Jawien et al., 2004)

1.7.2 Ldlr$^{-/-}$ mouse model of atherosclerosis

Originally *Ldlr*$^{-/-}$ mice were described as a model of human familial hypercholesterolemia (Fazio & Linton, 2001), but further studies established this line as a model of diet-induced atherosclerosis (Ishibashi, Goldstein, Brown, Herz, & Burns, 1994). On a normal chow diet *Ldlr*$^{-/-}$ mice have a 2-3-fold elevated plasma cholesterol level and begin to develop early atherosclerotic lesions around 6-7 months of age, but advanced lesions are normally not observed even further in life. However when *Ldlr*$^{-/-}$ mice are fed the Western diet (Daugherty, 2002) this leads to appearance of early atherosclerotic lesions already after 4-6 weeks of feeding, whereas intermediate lesions are observed between 12 and 16 weeks of feeding. More advanced lesions appear after prolonged feeding, between 16 and 20 weeks (Whitman, 2004).

1.8 Lipids and lipoproteins

Body lipids can be classified as the exogenous lipids that are resynthesized in the gut from dietary fats, and endogenous lipids, that are synthesized in the liver. Lipoproteins play a key role in the absorption and transport of dietary lipids from the small intestine, in the transport of endogenous lipids synthesized in the liver to peripheral tissues, as well as in the transport of lipids from peripheral tissues to the liver and intestine (reverse lipid transport). Another major function of lipoproteins is the transport of toxic hydrophobic and amphipathic compounds from the areas of invasion and infection (Feingold & Grunfeld, 2012). Lipoproteins are spherical macromolecular complexes composed of a hydrophilic coat of phospholipids, free cholesterol and specific amphipathic proteins, apoproteins and a hydrophobic core of non-polar lipids such as cholesterol esters and triglycerides (Hoofnagle & Heinecke, 2009) (Figure 5).

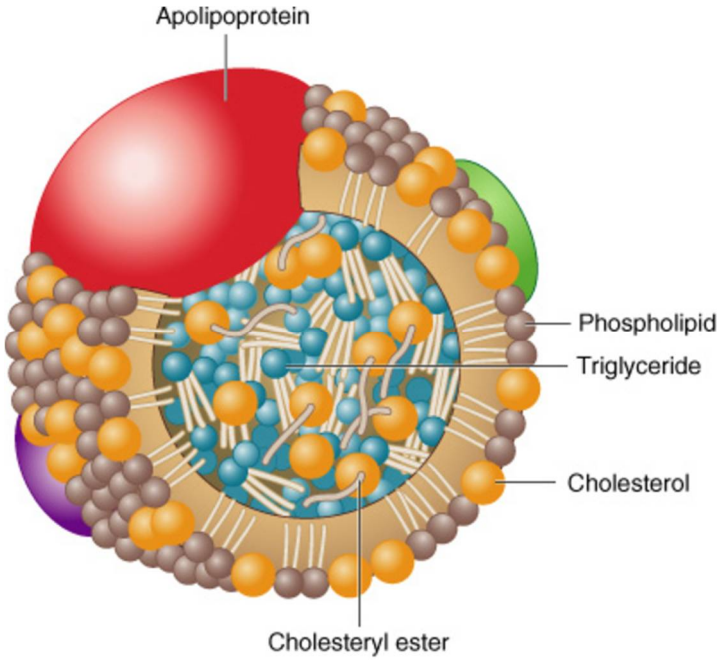

Figure 5. A schematic diagram representing the structure of lipoproteins.

1.9 Lipoprotein classification

The lipid-protein complexes vary in their size, composition and content of lipid and protein. Traditionally plasma lipoproteins are classified by density defined as the ratio of lipid to protein affecting their flotation during density gradient ultracentrifugation. Because lipids occupy a greater molecular volume then proteins and are therefore less dense, lipoproteins become progressively denser and smaller in size as the ratio of lipid to protein decreases (Lewis, 1973). Lipoproteins are divided into five major classes based on the relative contents of lipids and proteins: chylomicrons (CM), very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Figure 6) (Hegele, 2009).

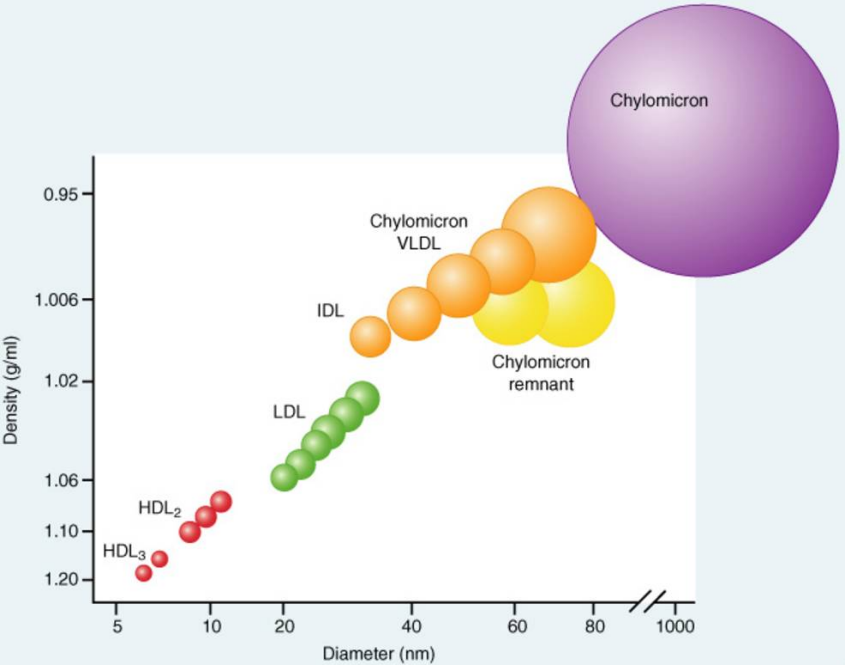

Figure 6. Relative size of plasma lipoproteins according to their density.

Besides density, lipoproteins are also classified according to their net charge. All lipoproteins exhibit a negative net charge, determined by both the apoprotein and lipid constituents (Sparks & Phillips, 1992). Based on the charge measured by their electrophoretic mobility lipoproteins can be separated into α, pre β, β and broad β lipoprotein classes. The electrophoretic mobility of a lipoprotein is mainly dependent upon its protein content: those with higher protein content will move faster in the agarose gel towards the anode and those with low protein content will have lower mobility. Thus, HDL are α, VLDL are pre β, LDL are β and IDL are broad beta lipoproteins (Lewis, 1973; Noble, 1968). In addition, lipoproteins can be classified on the basis of their immunological characteristics, particle size and by conferred principal apoproteins (Morita, 2016).

1.10 Structure and biochemical composition of LDL

LDL is a spheroidal particle with an average diameter of 22-28 nm and a density of 1.019-1.063 g/ml. Its' hydrophobic central core contains approximately 1600 molecules of cholesteryl esters and 170 molecules of triglycerides. The core is surrounded by a phospholipid-rich shell, which consists of 600 molecules of free cholesterol, approximately 700 phospholipid molecules and a small amount of lyso-phospholipids, gangliosides, sphingomyelin, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol (Esterbauer, Gebicki, Puhl, & Jurgens, 1992). Each LDL particle contains one molecule of apolipoprotein B 100 (ApoB) attached to the phospholipid-rich surface, that plays a crucial role in the interaction of the lipoprotein particle with the hepatocyte LDL receptor (LDLR), but not with other members of the LDLR family (Schumaker, Phillips, & Chatterton, 1994) (Esser, Limbird, Brown, Goldstein, & Russell, 1988).

1.11 Structure and function of apolipoproteins

Apolipoproteins are essential for lipoprotein structural assembly, secretion and function. Apolipoproteins have several major roles: they guide formation of the lipoproteins and support their structure; they act as ligands for lipoprotein receptors; and they serve as activators or inhibitors of enzymes involved in the metabolism of lipoproteins(Hoofnagle & Heinecke, 2009). Plasma apolipoproteins can be classified in two groups: nonexchangeable apolipoproteins such as ApoB 100, or ApoB48 and exchangeable apolipoproteins such as ApoE, ApoA-I, ApoA-II, ApoA-IV, ApoC-I, ApoC-II, or ApoC-III (Segrest, Jones, De Loof, & Dashti, 2001). Each lipoprotein class

has a characteristic apolipoprotein composition. One or more apolipoproteins (proteins or polypeptides) are present in each lipoprotein. The major apolipoproteins of HDL (α-lipoprotein) are designated as A class. The main apolipoproteins of LDL (β-lipoprotein) belong to the B class, represented by ApoB. They are also found in VLDL. Chylomicrons contain a truncated from of ApoB (B48) synthesized in the intestine, while a full-size ApoB is synthesized in the liver. ApoE is found in VLDL, HDL, chylomicrons and chylomicrons remnants (Stocker & Keaney, 2004).

1.12 ApoE and ApoB - lipoproteins most associated with atherosclerosis

1.12.1 Apolipoprotein E

ApoE, is a 34-kDa (299 amino acids) polymorphic multifunctional plasma apoprotein with a single O-linked glycan chain (Wernette-Hammond et al., 1989). In human plasma approximately 20% of all ApoE molecules are sialylated and a sialylation has been linked to their clearance rate (Millar, 2001).

ApoE is a ligand for the low-density lipoprotein receptor (LDLR) and LDLR-related protein (LRP). The majority of plasma ApoE is produced by the liver and macrophages but the protein is also synthesized by other organs such as the brain, kidneys and spleen (Boisvert, Spangenberg, & Curtiss, 1995). As a ligand for the receptor-mediated clearance of lipoproteins, ApoE is an important modulator of atherosclerosis. ApoE promotes the internalization of TG-rich lipoprotein remnants by hepatocytes, so the lack of it leads to lipoprotein accumulation (Schaefer et al., 1986). *ApoE* knockout mice have high levels of plasma cholesterol and spontaneously develop atherosclerosis (S. H. Zhang et al., 1992). Besides, in the atherosclerotic lesions ApoE directly

modifies the macrophage-mediated immune response that contributes to atherosclerosis (Curtiss & Boisvert, 2000; Perrey et al., 2001).

1.12.2 Apolipoprotein B

Apolipoprotein B (ApoB) is the main structural surface protein present in all beta-lipoproteins (chylomicrons, VLDL, IDL and LDL) in stoichiometry of one molecule of ApoB per lipoprotein. ApoB has two isoforms: ApoB100, a 4536-amino acid-secretory glycoprotein (550kDa) and ApoB48, a 2152-amino acid protein corresponding to the N-terminal 48% sequence of ApoB100 (Segrest et al., 2001). ApoB48 is produced in the proximal small intestine by tissue-specific mRNA splicing that generates a truncated form of ApoB. ApoB48 directs the formation of chylomicrons which main function is absorption and transport of lipids absorbed by the intestine. A small amount of *APOB* mRNA however escapes editing, resulting in a low level of ApoB100 present in the intestine (Kane, 1983). The full-length protein is constitutively synthesized in the liver by hepatocytes, where it assembles in VLDL. After VLDL are excreted and undergo lipolysis they become IDL and LDL (Kane, 1983).

Human ApoB100, one of the largest known monomeric proteins, is highly hydrophobic (Shelness & Sellers, 2001). Its folding requires assistance of chaperone proteins, post-translational lipidation, formation of disulfide bonds, and glycosylation. The fraction of ApoB100 that remains misfolded post-transnationally is eliminated through autophagy (Macri & Adeli, 1997). There are 8 disulfide bonds in ApoB100, 7 of them are found within the N-terminal 21% of its sequence (Yang et al., 1990). The amino acid sequence of ApoB100 contains 19 potential N-glycosylation sites, 17 of these are occupied by high-mannose and complex forms of

oligosaccharides (Taniguchi, Ishikawa, Tsunemitsu, & Fukuzaki, 1989) (Triplett & Fisher, 1978). Most of the ApoB100 N-glycans contain terminal sialic acid residues present in the α2-6 conformation. Six of the mature ApoB100 N-glycans are located close to the LDLR-binding region (Yang et al., 1986), however they do not play a significant role in LDL endocytosis (Shireman & Fisher, 1979). The potential function of ApoB100 glycans in post-hepatic secretion is still unknown.

1.13 Lipoprotein metabolism

Lipoprotein metabolism involves an exogenous pathway, endogenous pathway and reverse cholesterol transport.

1.13.1 Exogenous (dietary) pathway – chylomicron processing

Hydrolyzed dietary fats enter intestinal cells (enterocytes) via fatty acid transporters (Figure 7). Reconstituted TG are packaged with esterified cholesterol and ApoB48 into chylomicrons by the microsomal TG-transfer protein (MTTP) through a vesicular pathway (Hegele, 2009). In circulation, the nascent chylomicrons acquire ApoC and ApoE from plasma HDL in exchange for phospholipids. The acquisition of ApoC-II from HDL is essential to activate lipoprotein lipase (LPL) (Huff, Pollex, & Hegele, 2006). CM bind to membrane-bound LPL located on adipose and muscle tissues where the TG are hydrolyzed into free fatty acid (FFA). FFA can be further taken up by the liver, adipose tissue or by muscles. In the adipose cells FFA are re-processed into triacylglycerols and stored, whereas in the muscle, FFA are oxidized to provide energy (Morita, 2016). As the tissues absorb the fatty acids, CM progressively diminish in size and

become chylomicron remnants, where ApoE is exposed on the particle surface and the released ApoC is returned to plasma HDL. The ApoE mediate uptake of chylomicron remnants by the liver via the LDLR and LRP. The ApoC proteins are continuously recycled between chylomicrons and HDL (Morita, 2016).

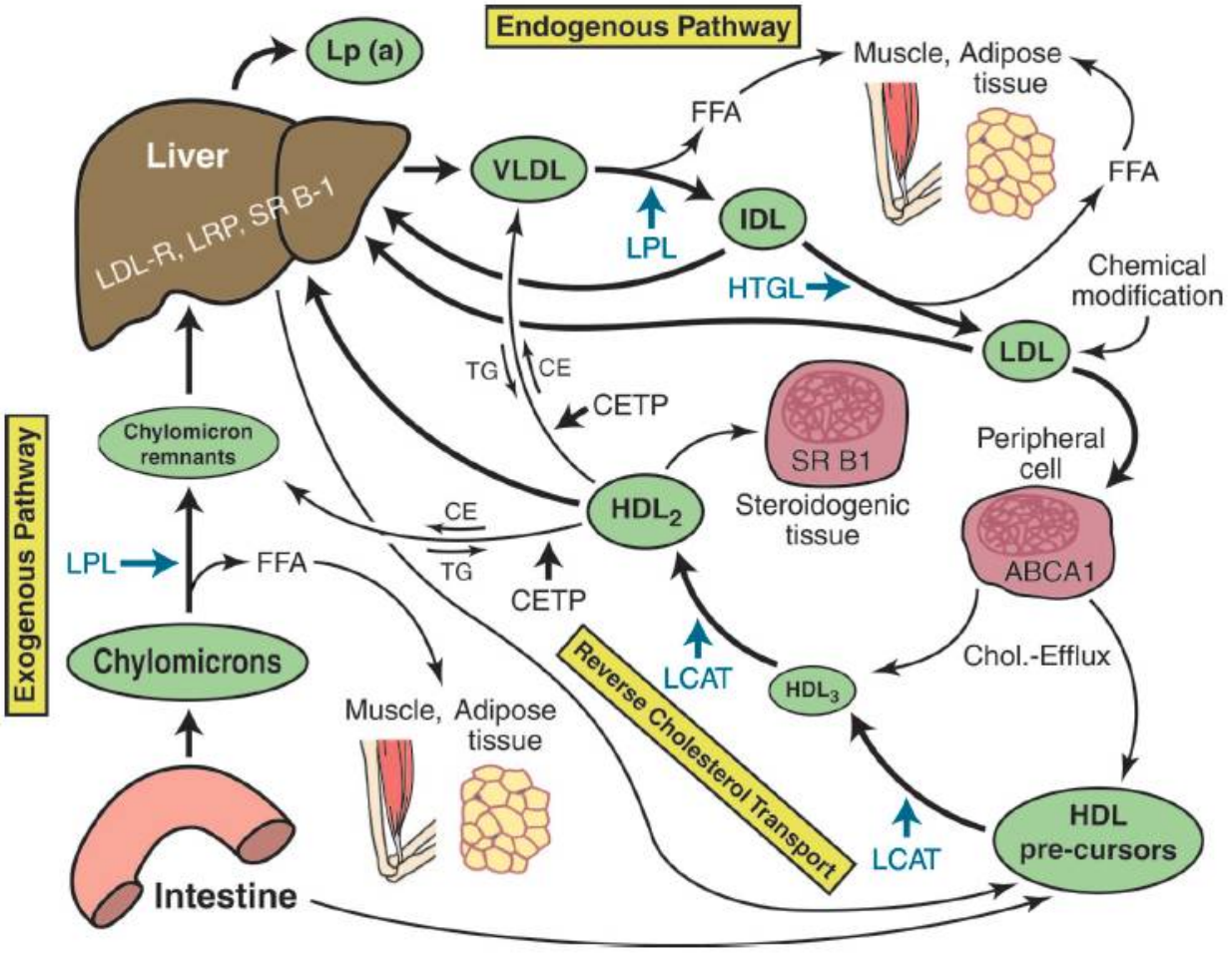

Figure 7. Major normal lipoprotein metabolic pathways.

ABCA1, ATP binding cassette transporter 1; CE, cholesterol ester; CETP, cholesteryl ester transfer protein; FFA, free fatty acid; HTGL, hepatic triglyceride lipase; IDL, intermediate-density lipoprotein; LCAT, lecithin: cholesterol acyltransferase; LDL-R, LDL receptor; Lp(a), lipoprotein(a); LPL, lipoprotein lipase; LRP, LDL-R–related protein; SR B1, scavenger receptor B1; TG, triglyceride (Kwan, Kronenberg, Beddhu, & Cheung, 2007).

1.13.2 Endogenous pathway – VLDL synthesis and processing. Conversion of VLDL to LDL

In hepatocytes TG assemble with cholesterol and ApoB into VLDL that are secreted via exocytosis (Figure 7). Like chylomicrons VLDL acquire ApoC and ApoE from HDL. VLDL are processed by the membrane-bound LPL of adipose and muscle tissues. LPL hydrolyses TG into FFA (Kwan et al., 2007) that are transported to the adipose cells for further synthesis and storage of TG or to the muscles to be oxidized and produce energy. After hydrolysis of the TG by LPL, VLDL release ApoC and phospholipids to HDL. VLDL considerably reduced in size forms IDL. IDL can be taken up by the liver or they can be further hydrolyzed by LPL, eventually loosing ApoE to form LDL (Morita, 2016). LDL are endocytosed by peripheral cells and hepatocytes through LDLR specifically recognizing ApoB. Approximately 75% of all LDL are absorbed by the liver. The remaining LDL are removed by other cells via LRP and other scavenger receptors (Brown & Goldstein, 1983).

1.13.3 Reverse cholesterol transport – role of HDL

HDL via ApoA-I and ApoA-IV mediate reverse cholesterol transport by interacting with ATP-binding cassette A1 (ABCA1) and ATP-binding cassette G1 (ABCG1) transporters on non-hepatic cells (Figure 7) (Morita, 2016). HDL remove excess cholesterol from peripheral cells and carry it to the liver, for conversion into bile salts. Precursors of HDL particles having a disk-shaped structures are secreted by the liver and intestine (Kwan et al., 2007). They acquire spherical shape while they absorb cholesterol from cell membranes and TG from other lipoproteins. The major apolipoprotein of HDL, ApoA-I activates lecithin-cholesterol acyltransferase (LCAT) that catalyzes the transfer of long-chain fatty acids from phospholipids to cholesterol forming cholesterol esters

to be further incorporated into HDL (Morita, 2016). After remodeling by the cholesterol ester transfer protein (CETP), that mediates exchange of cholesterol esters between lipoproteins, and by endothelial lipase, larger in size HDL particles enter hepatocytes via the scavenger receptor class B type I (SRB1) expressed in the liver and steroidogenic tissues (Acton et al., 1996). When HDL grow in size they also acquire multiple copies of ApoE, so they can further be taken up by the liver via LDLR (Bruce, Chouinard, & Tall, 1998).

1.14 LDL receptor pathway

The LDL receptor, LDLR is a 160 kDa glycoprotein present in all mammalian cell types (T. Yamamoto et al., 1984) that has affinity for two apolipoproteins, ApoB and ApoE (Willnow, 1997). LDLR plays a major role in cellular cholesterol homeostasis and also participates in a variety of regulatory modulations (Figure 8). LDL catabolism is primarily mediated through the hepatic LDLR. Following the internalization LDL degrade in the lysosomes, releasing LDL cholesterol. This suppresses expression of HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase, the key enzyme in the biosynthesis of cholesterol, and induces expression of LDLR. Thus, cholesterol content regulates the level of LDLR. Proprotein convertase subtilisin/kexin type 9 (PCSK9) diverts LDLR into the endosomal-lysosomal pathway for degradation (Seidah et al., 2003). Besides, LDL can suppress LDLR expression through the inhibition of the SREBP (sterol regulatory element-binding protein) pathway (Brown & Goldstein, 1999). A high level of cellular cholesterol activates the cholesterol-esterifying enzyme, cholesterol acyl-transferase (ACAT) so the excess of cholesterol can be stored as cholesteryl ester droplets in the cytoplasm (Brown, Dana, &

Goldstein, 1975). All these actions allow cells to regulate the amount of LDLR and level of cholesterol for their metabolic needs without causing cholesterol over accumulation.

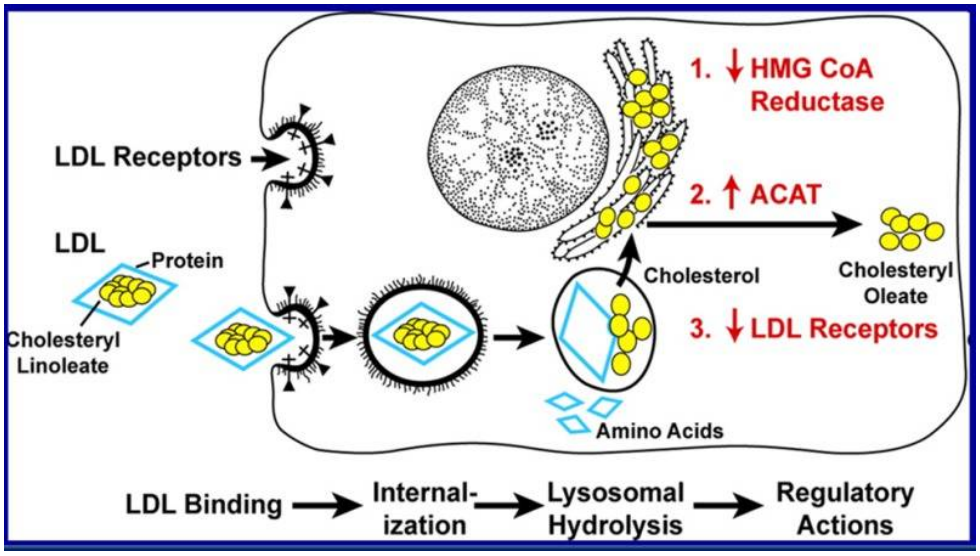

Figure 8. Sequential steps in the LDL receptor pathway.
HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A reductase; ACAT, cholesterol acyl-transferase. Modified from Brown and Goldstein (Brown & Goldstein, 1979).

1.15 LDL and atherosclerosis

A high level of cholesterol in LDL particles is a well-known risk factor for the development and progression of atherosclerosis (Bentzon, Otsuka, Virmani, & Falk, 2014; Keys, 1997; Martin, Hulley, Browner, Kuller, & Wentworth, 1986). However, 46% of first cardiovascular events occur in people with LDL levels at the normal range (Packard & Libby, 2008). Atherogenicity of LDL can be caused not only by the quantity, but also by certain properties of LDL, such as a small size, high density (Griffin, 1999) or their modification (Ahotupa, Suomela, Vuorimaa, & Vasankari, 2010). LDL are defined as lipoprotein particles with a density between 1.019 and 1.063 g/ml, however their composition is extremely heterogeneous and includes particles with different sizes, structures and chemical compositions (Atkinson, Deckelbaum, Small, & Shipley, 1977;

Fisher, 1972; Shen, Krauss, Lindgren, & Forte, 1981). Specifically, predominance of small dense LDL (d=1.044-1.060 g/ml) was associated with increased risk of CAD (Krauss, 1995).

Another plasma lipoprotein(a), Lp(a), that represents a subclass of LDL has been also associated with development of CAD (G. T. Jones et al., 2007). Lp(a) has as a protein moiety ApoB linked covalently to a single molecule of Apo(a) a specific multi-kringle protein of the plasminogen family (Figure 9) (Scanu, Lawn, & Berg, 1991). Lp(a) is unique to humans, apes, monkeys and hedgehog (Sabarinath & Appukuttan, 2015). The Apo(a) molecule is heavily glycosylated (glycans contribute to 28% of Apo(a) molecular mass (Fless, ZumMallen, & Scanu, 1986)) and linked to the apoprotein ApoB by a disulphide bond. It has been shown that Lp(a) is the dominant lipoprotein in human atherosclerotic plaques and its level increases with the plaque progression (Pepin, O'Neil, & Hoff, 1991; van Dijk et al., 2012).

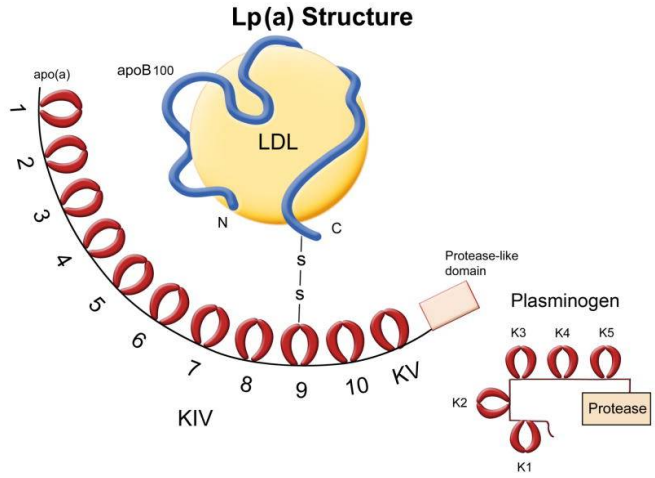

Figure 9. Lp(a) Structure.

Lp(a) consists of a LDL lipid core with ApoB attached by a disulfide bond to Apo(a). Apo(a) contains a variable number of kringle domains (KIV-1–10) that have a high homology to K4 of plasminogen, one KV kringle similar to K5 of plasminogen, and a proteolytic-like domain. KIV-2 is present in 2–50 copies, imparting extreme heterogeneity to Lp(a) (Hoover-Plow & Huang, 2013).

Biochemical modifications of ApoB contribute to LDL atherogenicity. There are many modifications that can occur with ApoB, such as: partial enzymatic hydrolysis (Bhakdi et al., 1995), chlorination or nitration (Heinecke, 1999), free radical oxidation of the polypeptide (Bruce et al., 1998), and modifications of ApoB glycan chains including their desialylation (Tertov et al., 1993). In macrophages, oxidized LDL (oxLDL) are taken up with the help of scavenger receptors such as CD36, SR-A1 and lectin-like oxLDL receptor-1 (LOX-1) (Gimbrone & Garcia-Cardena, 2013). Oxidation or acetylation of LDL in vitro converts them into ligands of scavenger receptors, but whether oxidation is the primary LDL modification in vivo is still unknown.

Another modification of LDL that may lead to atherosclerosis is desialylation (Orekhov, Tertov, & Mukhin, 1991). Fractions of ApoB-containing LDL with reduced content of sialic acids (Sia) were found in human blood (Bartlett & Stanley, 1998). Sia content of LDL in patients with atherosclerosis is lower than in healthy subjects (Ruelland, Gallou, Legras, Paillard, & Cloarec, 1993; Tertov et al., 1993). Desialylated LDL (desLDL) are rapidly taken up and accumulated by peripheral blood monocytes and by smooth muscle cells isolated from human arterial intima (Bartlett, Grewal, De Angelis, Myers, & Stanley, 2000).

Combined treatment with trypsin, cholesterol esterase, and neuraminidase transforms LDL into particles with a lipid structure, biological properties and composition similar to that of lipids extracted from atherosclerotic lesions (Bhakdi et al., 1995). Another study reported that enzymatic desialylation of LDL caused physical and chemical modifications in the structure of ApoB and resulted in accumulation of neutral lipids and cholesteryl esters in human aortic intimal cells (Tertov et al., 1992). DesLDL have exposed Gal residues (Taniguchi et al., 1989) which makes them a high affinity ligand for lectin receptors such as the asialoglycoprotein receptor (Lee et al.,

1983). Importantly, lipopolysaccharide-stimulated macrophages expressing increased amounts of Gal/GalNAc-specific lectin on their surface (Grewal, Bartlett, Burgess, Packer, & Stanley, 1996) showed high uptake rates of desLDL. Desialylated LDL can also bind scavenger receptors, and cellular surface proteoglycans (Camejo, Lopez, Lopez, & Quinones, 1985). DesLDL are smaller in size, higher in density, have lower negative charge and reduced affinity to the LDL receptor (Orekhov et al., 1992). Besides, desialylation of LDL can lead to their aggregation, which is a key factor for intracellular lipid accumulation (Musliner, McVicker, Iosefa, & Krauss, 1987).

1.16 Foam cell formation

Circulating monocytes differentiate into macrophages after migration into the intima. In macrophages, a range of genes related to lipid metabolism, such as *LDLR*, *PCSK9*, *APOB* play critical roles in maintaining normal cholesterol homeostasis (Kjolby, Nielsen, & Petersen, 2015; Lamon-Fava, 2013; Nozue et al., 2016).

Atherogenic modifications of LDL prevent their recognition by (or reduce their affinity for) the low-density lipoprotein receptor (LDLR) (Tabas, 2002). As a result, modified LDL become recognized by another group of receptors – scavenger receptors – that are expressed on the cell surface of macrophages. Scavenger receptors identified in macrophages include: scavenger receptor AI/II (SR-AI/II), scavenger receptors cluster of differentiation 36 (CD36), LOX-I, SR-BI (Levitan, Volkov, & Subbaiah, 2010). Scavenger receptor type A (SR-A) was shown to be specific for the recognition and the uptake of acetylated and oxidized LDL (Kodama et al., 1990). Cytokines and modified LDL themselves induce expression of scavenger receptors. It is also increased during differentiation of monocytes to macrophages (Ye et al., 2009). Unlike the LDLR,

scavenger receptors do not undergo negative feedback regulation in response to intracellular cholesterol accumulation (Moore & Freeman, 2006). LDL, recognized via scavenger receptors are endocytosed and lysosomes loaded with LDL accumulated in the cytoplasm, resulting in the formation of the foam cells (Morita, 2016). Some lipid-loaded macrophages can leave the arterial wall, thus taking away the lipids from the artery. Otherwise, they contribute to the consequential growth of atheroma (X. H. Yu et al., 2013).

1.17 Sialic acids

Sialic acids (Sia) are negatively charged N-or O-acyl derivatives of a 9-carbon sugar called neuraminic acid (5-amino- 3, 5-dideoxy-2 nonulosonic acid) (Figure 10) (X. Chen & Varki, 2010). Sia are common terminal sugar components of the oligosaccharide chains of glycoproteins and glycolipids.

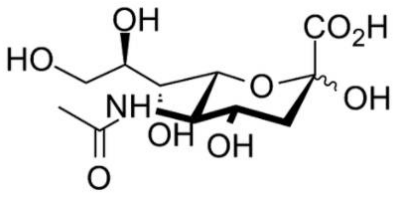

Figure 10. Chemical structure of the most common human sialic acid, N-acetyl neuraminic acid

Sia are involved in a surprising variety of biological processes, including conformational stabilization of molecules, regulation of cell surface charge, cell recognition, cell differentiation, interaction, migration, adhesion, immune response and metastasis (X. Chen & Varki, 2010; Drake, Balbis, Wu, Bergeron, & Posner, 2000). The majority of soluble secreted and lysosomal proteins contain Sia as part of their glycan chains, and this modification extends their half-life. Sia are also

present on the surface of erythrocytes and platelets and the level of sialylation determines the life span of these cells in circulation (Aminoff, Bell, Fulton, & Ibgebrigtsen, 1976). It is thought that sialylation provides for the negative surface charge of erythrocytes which is important for electrostatic repulsion between the cells (Chien, 1986). Members of the Siglecs (sialic acid binding immunoglobulin-like lectins) superfamily mediate intracellular interactions which contribute to the scavenging function of macrophages, pathogen uptake and antigen presentation (Kahn, 2000). Glycosynapses enriched in sialylated glycoproteins and glycolipids mediate cell signaling and participate in cell adhesion, motility and growth (Brunzell et al., 1976). Cancer cells have long been recognized to have a significant over-expression of Sia on the cell surface (Bevan et al., 2000). Lipid- and protein-bound Sia are elevated in plasma from cancer patients (Contreres, Faure, Baquiran, Bergeron, & Posner, 1998) and linked with acute phase condition and chronic disease (C. Yu et al., 2002). The majority of Sia in the blood are bound to proteins and glycolipids. In particular Sia associated with lipoproteins are attached to the carbohydrate chains of apolipoproteins (Taniguchi et al., 1989) and ganglioside molecules (mainly GM3 ganglioside) present in the lipid core. The content of Sia decreases from VLDL to LDL and further to HDL (Harada, Carvalho, Passarelli, & Quintao, 1998). Glycosylation plays an important role in the biology of LDL as all LDL particles contain sialylated oligosaccharides (Millar, 2001; Orekhov et al., 1991). Every ApoB molecule in human plasma LDL contains approximately 12-14 Sia residues (Millar, 2001). Apolipoproteins CII and CIII, which are found to VLDL and are responsible for the interaction of VLDL with lipoprotein lipase are also heavily sialylated with functional consequences (Mauger, Couture, Bergeron, & Lamarche, 2006). Sialic acid is also largely present in the glycan chains of the mature LDLR (Cummings et al., 1983) and is responsible

for the net negative charge of this protein (Goldstein, Brown, Anderson, Russell, & Schneider, 1985). Importantly sialylation of LDLR plays an important role in its interaction with LDL, evidenced by the fact that treatment of fibroblasts and endothelial cells with bacterial neuraminidase had significantly altered LDL binding, internalization and degradation by these cells(Sprague, Moser, Edwards, & Schwartz, 1988). PCSK9 also binds to the highly glycosylated ligand-binding region of LDLR, thus adding further potential implications for sialylation in LDLR regulation and degradation (T. Yamamoto, Lu, & Ryan, 2011). Several studies showed that a high level of free sialic acid in human plasma is associated with CAD and atherosclerosis (Knuiman, Watts, & Divitini, 2004; Wakabayashi, Sakamoto, Yoshimoto, & Kakishita, 1994). Patients with atherosclerosis contain LDL in their blood with a 2.5-5 fold lower sialic acid content compared to that of healthy subjects, at the same time there was no difference in protein and lipid content between the two groups of patients (Orekhov et al., 1991). The sialylation of ApoB has been directly implicated into the risk of atherosclerosis (Mel'nichenko et al., 2005; Sobenin, Tertov, Orekhov, & Smirnov, 1991).

1.18 Neuraminidases

Neuraminidases (sialidases) are enzymes that catalyze removal of terminal Sia residues from glycoproteins, oligosaccharides, and sialylated glycolipids. The mammalian genomes contain four genes, which encode members of the neuraminidase family (*NEU1-NEU4* in humans). These enzymes have different, yet overlapping tissue expression, intracellular localization and substrate specificity (Milner et al., 1997; Monti et al., 2000; Monti, Preti, Nesti, Ballabio, & Borsani, 1999; Seyrantepe et al., 2004) (Table 1). *NEU1* is ubiquitously expressed with

the highest levels in the kidney, pancreas, skeletal muscle, liver, lungs, placenta and brain (Bonten, van der Spoel, Fornerod, Grosveld, & d'Azzo, 1996; Milner et al., 1997; Pshezhetsky et al., 1997). In these tissues *NEU1* generally shows 10–20 times higher expression than *NEU3* and *NEU4*, and ~10^3-10^2 higher expression than *NEU2* (Yamaguchi et al., 2005). *NEU2* is expressed predominantly in muscle tissues (Sato & Miyagi, 1996). *NEU3* has the highest expression in the adrenal gland, skeletal muscle, heart, testis and thymus (Monti et al., 2000; Wada et al., 1999). *NEU4* has the highest expression in brain, skeletal muscle, heart, placenta and liver (Comelli, Amado, Lustig, & Paulson, 2003; Seyrantepe et al., 2004; Yamaguchi et al., 2005). In the cell, Neu1 protein is localized at the lysosomal and plasma membranes (Lukong et al., 2001; Vinogradova et al., 1998); cytosol (Koda, Kijimoto-Ochiai, Uemura, & Inokuchi, 2009; Miyagi & Tsuiki, 1985; Monti, Preti, Rossi, Ballabio, & Borsani, 1999; Tringali et al., 2004). Neu3 is a membrane-associated protein localized in the caveolae microdomains of plasma, endosomal and lysosomal membranes (Y. Wang et al., 2002; Zanchetti et al., 2007); Neu4 protein is present on the cell membrane, mitochondria, endoplasmic reticulum (Bigi et al., 2010; Monti et al., 2004; Seyrantepe et al., 2004; Yamaguchi et al., 2005). Since neuraminidases show partially overlapping substrate specificities in vitro, their distinct tissue and subcellular distribution may be key to their biological roles. Neu1 is active only in the form of a complex with carboxypeptidase Cathepsin A (CathA) and β-galactosidase, however, the exact mechanisms of this activation and intracellular transport remain unclear (Pshezhetsky & Ashmarina, 2001). Neu1 shows activity primarily against sialylated glycopeptides and oligosaccharides with much lower activity against gangliosides. The enzyme is involved in the lysosomal catabolism of the above conjugates (Dorland et al., 1978; Michalski, Strecker, & Fournet, 1977; Strecker et al., 1977; van Pelt, Kamerling, Vliegenthart,

Verheijen, & Galjaard, 1988; Yoshino et al., 1990), but growing experimental evidence demonstrates that Neu1 also participates in regulation of cell signaling by desialylating plasma membrane receptors (Figure 11) (Dridi et al., 2013; Pshezhetsky & Hinek, 2011).

Table 1. General properties of four mammalian neuraminidases.

	Neu1	Neu2	Neu3	Neu4
Major subcellular localization	Lysosomes Plasma membranes	Cytosol	Plasma membranes Endosomes	Lysosomes[a], Mitochondria and ER[a]
Preferred substrates	Oligosaccharides Glycopeptides	Oligosaccharides Glycoproteins Gangliosides	Gangliosides	Oligosaccharides Glycoproteins Gangliosides
Optimal pH	4.4–4.6	6.0–6.5	4.5–4.7	4.5–4.7
Total amino acids **Human** **Mouse**	 415 409	 380 379	 428 418	 496 (484)[b] 501 (478)[b]
Chromosomal location **Human** **Mouse**	 6p 21.3 17	 2q 37 1	 11q 13.5 7	 2q 37.3 10
Proposed major functions	Lysosomal catabolism Deficiency causes sialidosis Exocytosis Immune function Phagocytosis Elastic fiber assembly	Myoblast differentiation Neuronal differentiation	Neuronal differentiation Apoptosis Adhesion	Neuronal differentiation Apoptosis Adhesion

[a] Different compartment for subcellular localization of human Neu4, have been reported based on transient transfection of tagged protein in cultured cells.

[b] Human and mouse Neu4 proteins exist in two isoforms, long (L, Neu4a) and short (S, Neu4b), which differ by the presence of an N-terminal amino acid peptide (12 amino acid residues for human enzymes and 23 amino acid residues for the mouse). Adopted from (Miyagi & Yamaguchi, 2012)

Genetic defects in the *NEU1* gene in humans cause severe metabolic disease sialidosis characterized by lysosomal storage of glycoconjugates, skeletal abnormalities, hepatomegaly, neurodegeneration and neuroinflammation resulting in progressive myoclonus, blindness and cognitive impairment (Pshezhetsky et al., 1997). After discovery of the human *NEU1* gene about 40 mutations have been identified and characterized in sialidosis patients (Bonten et al., 1996; Pattison, Pankarican, Rupar, Graham, & Igdoura, 2004; Pshezhetsky et al., 1997; Ranganath, Sharma, Danda, Nandineni, & Dalal, 2012; Seyrantepe et al., 2003). Neu1 has been reported to be a lysosomal membrane protein with a C-terminal lysosomal targeting motif (Lukong et al., 2001), but another study showed that its association with cathepsin A (CathA) is necessary for trafficking of Neu1 to the lysosome (Galjart et al., 1991). The N-linked glycans of Neu1 are important for its stability and activity, and CathA can counteract against defects in N-glycosylation (D. Wang, Zaitsev, Taylor, d'Azzo, & Bonten, 2009). Defects in CathA in human patients result in secondary Neu1 deficiency and cause the severe lysosomal storage disease galactosialidosis clinically and biochemically resembling sialidosis (Goldberg et al., 1971; Okamura-Oho, Zhang, & Callahan, 1994).

Neu2 is active against α2-3-sialylated oligosaccharides, glycopeptides and gangliosides (Miyagi & Tsuiki, 1985). Neu3 is a crucial regulator of transmembrane signaling (Yamaguchi, Hata, Wada, Moriya, & Miyagi, 2006) and has been implicated in regulation of cell transformation, differentiation and migration (Y. Wang, Yamaguchi, Shimada, Zhao, & Miyagi, 2001) neuritogenesis, carcinogenesis and apoptosis as well as insulin signaling (Y. Wang et al., 2001). Neu4 is active against all types of sialylated glycoconjugates including oligosaccharides, glycoproteins and gangliosides (Hasegawa et al., 2007; Seyrantepe et al., 2004; Shiozaki,

Yamaguchi, Takahashi, Moriya, & Miyagi, 2011a). In the mouse brain, Neu4 is involved in regulation of neuronal cell differentiation(Chavas et al., 2005).

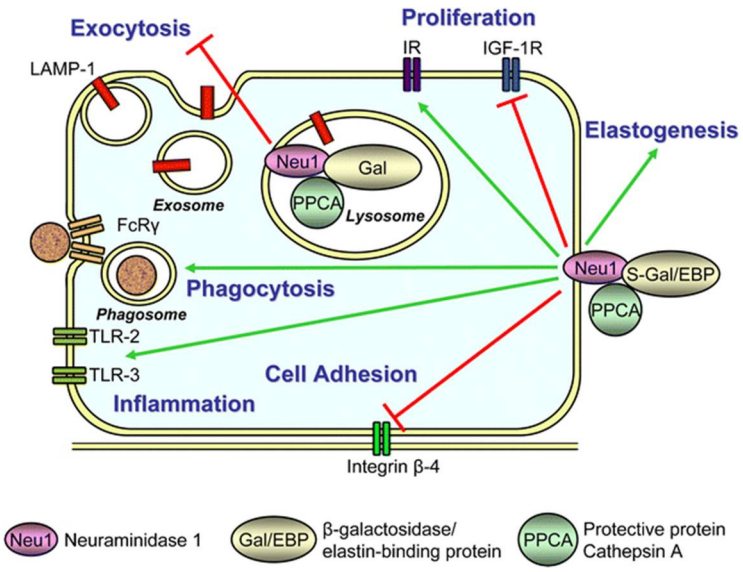

Figure 11. Proposed roles of Neu1 in regulation of cell signaling.
Components of the multienzyme lysosomal complex, neuraminidase 1 (Neu1), protective protein/cathepsin A (PPCA) and β-galactosidase (Gal), are sorted from the trans-Golgi network to the endosomal pathway where they participate in the lysosomal catabolism of glycoconjugates. Neu1-PPCA-Gal complex is also sorted to the vesicles destined to the plasma membrane. Neu1 and CathA form the elastin receptor targeted to the plasma membrane and involved in extracellular assembly of elastic fibers. Neu1 desialylates and activates receptors for phagocytosis (FcRγ), inflammation (TLR-1 and TLR-2) and cell proliferation/glucose uptake (IR). It also desialylates and inhibits receptors for cell adhesion (Integrin β-4), exocytosis (LAMP-1) and cell proliferation (IGF-1R) (Pshezhetsky & Hinek, 2011).

Role of sialylation in immunity has been well studied (Varki & Gagneux, 2012). It has been shown that endogenous neuraminidase activity increases in the immune cells during cell activation (X. P. Chen, Enioutina, & Daynes, 1997). Neuraminidase expression is involved in the secretion of numerous inflammatory factors, such as IL-4 (X. P. Chen et al., 1997). Lukong et al., showed that Neu1 is increased on the surface of activated T-cells. Upregulation of Neu1 is involved in the differentiation of monocytes into macrophages, as well as the differentiation of THP-1 monocytes (Lambre, Greffard, Gattegno, & Saffar, 1990; Liang et al., 2006; Lukong et al., 2001). Importantly the induced enzyme is targeted mainly to the cell membrane (Liang et al.,

2006). The cell surface Neu1 activates phagocytosis in macrophages and dendritic cells through desialylation of FcγR (Seyrantepe et al., 2010). Through desialylation Neu1 controls ligand-induced TLR activation (Abdulkhalek et al., 2011; Amith et al., 2009). Moreover, neuraminidases affect surface glycoproteins on endothelial cells leading to increased adherence of neutrophils (Feng et al., 2011).

Neu1 has been linked to insulin receptor activation and glucose metabolism in mice (Dridi et al., 2013) and Neu1 activity is altered in epididymal fat and livers of obese and diabetic mice (Natori, Ohkura, Nasui, Atsumi, & Kihara-Negishi, 2013). Increased erythrocyte aggregation caused by high neuraminidase activity was also found to be associated with the risk of CAD (Chien, 1986).

The chemical inhibitor of neuraminidases 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) is active on all human and mammalian isoforms of neuraminidases and can be used to down-regulate neuraminidase activity (Y. Zhang et al., 2013). However, DANA has a low micromolar IC_{50} against all human neuraminidase (Burmeister, Henrissat, Bosso, Cusack, & Ruigrok, 1993). Modified analogs of DANA, however, show higher inhibition activity and moderate selectivity between different neuraminidases (Y. Zhang et al., 2013). For example, a recent study showed that a glycerol side-chain pocket in the active side of the neuraminidase can potentially be a target for the design of selective isoenzyme inhibitors (Y. Zhang et al., 2013).

1.19 Mouse models of neuraminidase deficiency

Three complete neuraminidase KO mice (*Neu1*, *Neu3* and *Neu4* KO) and 2 mice with partial Neu1 deficiency have been reported in the literature. The *Neu1* KO mice develop a

systemic disease that closely mimics the human sialidosis (de Geest et al., 2002). These mice have growth retardation, splenomegaly, severe neurologic deterioration and premature death (Zanoteli et al., 2010). Also they have progressive disruption of muscle fibers associated with alterations of extracellular matrix components and infiltration by connective tissue (Andrews, 2000). Further studies in the *Neu1* KO model linked Neu1 with regulation of lysosomal exocytosis. Cells isolated from Neu1-deficient mice exhibited a significant increase in docked lysosomes on the plasma membrane (Yogalingam et al., 2008), suggesting that lysosomal exocytosis is up-regulated in Neu1-deficient cells leading to increased secretion of lysosomal enzymes into the extracellular space and abnormal remodeling of the plasma membrane. In our laboratory, the mouse model with 10–15% of residual Neu1 activity in tissues (*CathA*$^{S190A\text{-}Neo}$ mice) was generated by inserting a *neo* cassette into an exon of the *CathA* (*Ctsa*) gene (Seyrantepe et al., 2010). This caused a hypomorphic affect drastically reducing CathA expression and causing partial Neu1 deficiency. The residual Neu1 activity level in the *CathA*$^{S190A\text{-}Neo}$ mice was sufficient to prevent lysosomal storage so mice did not show neurological symptoms of sialidosis. However, reduction of Neu1 in the immune cells affected the capacity of the cells to engulf bacteria or to produce cytokines (Liang et al., 2006).

The *Neu3* KO mice showed no abnormalities in their growth, development, behavior, or fertility but had more progressive induced colon carcinogenesis, suggesting that *Neu3* is involved in inflammation-dependent tumor development (Yamaguchi et al., 2012).

Neu4 KO mice generated in our laboratory showed normal growth, lifespan and fertility (Seyrantepe, Canuel, et al., 2008). However, these mice had increased levels of gangliosides, ceramide, cholesterol and fatty acids in the brain, liver, lungs, and spleen. They also had

lysosomal storage bodies in lung macrophages, spleen macrophages and lymphocytes (Seyrantepe, Canuel, et al., 2008).

1.20 Hypothesis and objectives

Atherosclerosis is a leading cause of death and loss of productive life years worldwide. LDL levels correlate with the risk of cardiovascular events in human populations, and augment individual susceptibility to atherosclerosis and its complications. Lowering LDL levels by statins compose the current mainstay of therapeutic management of atherosclerosis diminish the likelihood of atherosclerotic events (J. C. Wang & Bennett, 2012). At the same time, in CAD statins only benefit ~35% of patients (Arsenault, Kritikou, & Tardif, 2012). Antioxidant supplements such as vitamins E and C alone provided no benefit to CAD patients (Steinberg & Witztum, 2002). Despite experimental evidence that inflammation has a key role and transduces the effects of many known risk factors for atherosclerosis, many traditional anti-inflammatory therapies (glucocorticoids, non-steroidal anti-inflammatory drugs) do not improve cardiovascular outcomes, and some may even aggravate atherosclerotic events. The above evidence underlines the necessity of further insight into atherosclerosis leading to new clinical applications. The major role in atherogenesis is played by modified LDL, which have a longer circulation time due to a reduced affinity to LDL receptors (Tertov et al., 1992) and can provoke a cascade of responses that lead to disease in a previously unaffected (healthy) artery. Besides oxidation atherogenic LDL can be produced by desialylation, i.e. removal of terminal sialic acid residues from their glycan chains (Williams & Tabas, 1998). Sialic acid (Sia) constitutes 10% of the total carbohydrate content of the LDL surface glycoprotein, apolipoprotein B 100 (ApoB). Desialylation of LDL by

neuraminidases causes physical and chemical modifications in the ApoB structure, increases its affinity for the scavenger asialoglycoprotein receptors and cellular surface proteoglycans in macrophages and results in accumulation of neutral lipids and cholesteryl esters in human aortic intimal cells and in mouse peritoneal macrophages (Grewal et al., 1996; Tertov et al., 1992). In contrast to oxidation, desialylation is a naturally occurring modification of LDL. A high blood level of free Sia is an indication of atherosclerosis and CAD (Lindberg, 2007). Atherosclerotic lesions are frequently found in low-sialylated areas of aortic endothelium, removal of Sia from the intima of aorta by neuraminidases increases the adhesion of circulating platelets as well as the uptake of LDL (Gorog & Born, 1983).

All above data allow us to hypothesize that increased neuraminidase levels can influence predisposition to atherosclerosis and CAD in the human population potentially through desialylation of LDL occurring in the arterial wall. We suggest that an increased level of neuraminidases constitutes a risk factor/biomarker for atherosclerosis, which can identify novel potential targets for treatment of CAD.

To address this hypothesis, the specific aims of the project were: to characterize the substrate specificity of mammalian neuraminidases; to test that LDL are desialylated *in vivo* by the members of the neuraminidase family; to study the effect of desialylation on LDL uptake by macrophages as well as on LDL incorporation into the arterial walls of live mice; to study atherosclerosis progression in atherosclerotic mouse models deficient in individual neuraminidases. The results of these studies are presented in the following chapters.

Chapter 2. Structural basis for substrate specificity of mammalian neuraminidases (Published paper)

Structural Basis for Substrate Specificity of Mammalian Neuraminidases

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2.1 Abstract

The removal of sialic acid (Sia) residues from glycoconjugates in vertebrates is mediated by a family of neuraminidases (sialidases) consisting of Neu1, Neu2, Neu3 and Neu4 enzymes. The enzymes play distinct physiological roles, but their ability to discriminate between the types of linkages connecting Sia and adjacent residues and between the identity and arrangement of the underlying sugars has never been systematically studied. Here we analyzed the specificity of neuraminidases by studying the kinetics of hydrolysis of BODIPY-labeled substrates containing common mammalian sialylated oligosaccharides: 3'Sia-LacNAc, 3'SiaLac, $SiaLe_x$, $SiaLe_a$, $SiaLe_c$, 6'SiaLac, and 6'SiaLacNAc. We found significant differences in substrate specificity of the enzymes towards the substrates containing α2,6-linked Sia, which were readily cleaved by Neu3 and Neu1 but not by Neu4 and Neu2. The presence of a branching 2-Fuc inhibited Neu2 and Neu4, but had almost no effect on Neu1 or Neu3. The nature of the sugar residue at the reducing end, either glucose (Glc) or *N*-acetyl-D-glucosamine (GlcNAc) had only a minor effect on all neuraminidases, whereas core structure (1,3 or 1,4 bond between D-galactose (Gal) and GlcNAc) was found to be important for Neu4 strongly preferring β3 (core 1) to β4 (core 2) isomer. Neu3 and Neu4 were in general more active than Neu1 and Neu2, likely due to their preference for hydrophobic substrates. Neu2 and Neu3 were examined by molecular dynamics to identify favorable substrate orientations in the binding sites and interpret the differences in their specificities. Finally, using knockout mouse models, we confirmed that the substrate specificities observed *in vitro* were recapitulated in enzymes found in mouse brain tissues. Our data for the

first time provide evidence for the characteristic substrate preferences of neuraminidases and their ability to discriminate between distinct sialoside targets.

2.2 Introduction

Glycoproteins and glycolipids containing sialic acid (Sia) are found in abundance in mammalian cells, forming a dense array that covers the cell plasma and lysosomal membranes with complex sialylated structures (Cohen & Varki, 2010) often in a form of dynamic microdomains. The majority of plasma membrane-associated, secreted or lysosomal proteins contain Sia as part of their glycan chains and this modification extends their half-life (Byrne, Donohoe, & O'Kennedy, 2007). In mammals, the content of Sia strongly depends on the cell and tissue type, and changes significantly during development (C. J. Jones, Aplin, Mulholland, & Glasser, 1993).

Due to their diverse physical and chemical properties, Sia are involved in a surprising variety of biological processes (Kelm & Schauer, 1997). The most important role of Sia is the modulation of recognition events. Sia are well known as commonly exploited ligands for virus, bacteria, and protozoan pathogens. Sia also function as crucial recognition markers in multicellular organisms where they mediate a variety of biological phenomena, including cell differentiation, interaction, migration, adhesion, and metastasis (Allende & Proia, 2002; Kelm & Schauer, 1997; Lehmann, Tiralongo, & Tiralongo, 2006). Members of the sialic acid binding immunoglobulin-like lectin (Siglec) superfamily mediate intracellular interactions which contribute to the scavenging function of macrophages, pathogen uptake and antigen presentation (Varki & Angata, 2006). Glycosynapses mediate cell signaling and participate in

processes such as cell adhesion, motility and growth (Regina Todeschini & Hakomori, 2008). Cancer cells have long been recognized to have a significant over-expression of Sia on the cell surface [*e.g.(Babal, Janega, Cerna, Kholova, & Brabencova, 2006; Berbec, Paszkowska, Siwek, Gradziel, & Cybulski, 1999; Brooks & Leathem, 1998; Feijoo-Carnero et al., 2004)*]. Lipid- and protein-bound Sia are elevated in plasma from cancer patients (Basoglu et al., 2003; Berbec et al., 1999; Rajpura, Patel, Chawda, & Shah, 2005; Romppanen, Haapalainen, Punnonen, & Penttila, 2002; Uslu, Taysi, Akcay, Sutbeyaz, & Bakan, 2003) and linked with acute phase conditions and chronic disease [*e.g.(Herve, Duche, & Jaurand, 1998; Iijima, Shiba, Kimura, Nagai, & Iwai, 2000)*].

In mammals, the synthesis of sialoglycoconjugates is performed by a family of 20 sialyltransferases that catalyze the transfer of Sia from cytidine monophosphate-Sia to an acceptor carbohydrate (Takashima, 2008), whereas the removal of Sia by enzymatic cleavage is mediated by neuraminidases, represented in vertebrates by four gene families *(NEU1-4)*. These enzymes have different, yet overlapping tissue expression, intracellular localization and substrate specificity.

NEU1 is ubiquitously expressed with the highest levels in kidney, pancreas, skeletal muscle, liver, lungs, placenta and brain (Bonten et al., 1996). In these tissues, *NEU1* generally shows 10–20 times higher expression than *NEU3* and *NEU4*, and ~10^3-10^2 higher expression than *NEU2 (Yamaguchi et al., 2005)*. *NEU3* has the highest expression in adrenal gland, skeletal muscle, heart, testis and thymus (Monti et al., 2000; Wada et al., 1999). *NEU4* has the highest expression in brain, skeletal muscle, heart, placenta and liver (Comelli et al., 2003; Seyrantepe et al., 2004; Yamaguchi et al., 2005). In the cell, Neu1 is localized at the lysosomal and plasma membranes (Lukong et al., 2001; Vinogradova et al., 1998). Neu2 is a soluble protein found in the

cytosol (Koda et al., 2009; Miyagi & Tsuiki, 1985; Monti, Preti, Rossi, et al., 1999; Tringali et al., 2004). Neu3 is a membrane-associated protein localized in the caveolae microdomains of plasma, endosomal and lysosomal membranes (Y. Wang et al., 2002; Zanchetti et al., 2007). The *NEU4* gene is spliced in 2 different forms differing in the first 12 N-terminal amino acids (Monti et al., 2004; Yamaguchi et al., 2005). The short isoform was found predominantly on the ER membrane (Monti et al., 2004; Yamaguchi et al., 2005), whereas the long form is targeted both to mitochondria (Bigi et al., 2010; Yamaguchi et al., 2005) and lysosomes (Seyrantepe et al., 2004).

Previous studies have defined some biological functions for the four mammalian neuraminidases. Neu1 is crucial for the lysosomal catabolism of sialylated glycoconjugates (Dorland et al., 1978; Michalski et al., 1977; Strecker et al., 1977; van Pelt et al., 1988; Yoshino et al., 1990), but also participates in regulation of cell signaling through desialylation of plasma membrane receptors (Dridi et al., 2013). In contrast to other neuraminidases, Neu1 is active only in a complex with the lysosomal protease, cathepsin A (CathA) (Pshezhetsky & Ashmarina, 2001). Genetic deficiency of Neu1 in humans results in a severe metabolic disease, sialidosis (MIM #256550) (Pshezhetsky et al., 1997). In addition, genetic deficiency of *CATHA* results in the secondary deficiencies of Neu1 and lysosomal β-galactosidase and causes the lysosomal storage disorder, galactosialidosis (MIM #256540) (d'Azzo, Andria, Strisciuglio, & Galjaard, 1995; Okamura-Oho et al., 1994). Both disorders manifest clinically with skeletal and gait abnormalities, progressive impaired vision, ataxia, seizures and myoclonus syndrome. The biological role of Neu2 remains unknown, but the enzyme has been linked to the alteration of cytoskeletal functions during myoblast differentiation as well as oncogenesis (Albohy, Li, Zheng, Zou, & Cairo, 2010; Albohy, Mohan, Zheng, Pinto, & Cairo, 2011; Sandbhor et al., 2011; Y. Zhang et al., 2013).

Neu3 is a crucial regulator of transmembrane signaling (Kirschner et al., 2008) and is implicated in regulation of neurogenesis, carcinogenesis and apoptosis as well as in insulin signaling (Y. Wang et al., 2001). Neu4 participates in lysosomal and mitochondrial ganglioside catabolism (Hasegawa et al., 2007; Seyrantepe et al., 2004; Shiozaki et al., 2011a) and the regulation of neuronal cell differentiation (Chavas et al., 2005).

Although there is a diverse array of biological functions ascribed to the four members of the mammalian neuraminidase family, it is not clear if this is a result of different subcellular or tissue localization or the distinct substrate specificities of each isoenzyme. Previous studies of mammalian neuraminidase substrate specificity have mostly relied on their *in vitro* activities against major classes of sialoglycoconjugates: glycolipids, glycoproteins and free oligosaccharides. It has been reported, in particular, that Neu1 is active primarily against sialylated glycopeptides and oligosaccharides with lower activity against gangliosides; Neu3 requires a hydrophobic aglycone(Sandbhor et al., 2011), giving it a preference for gangliosides; whereas, Neu2 and Neu4 are active against all types of sialylated glycoconjugates including oligosaccharides, glycoproteins and gangliosides (Monti et al., 2004; Monti, Preti, Rossi, et al., 1999; Seyrantepe et al., 2004). At the same time, the ability of the enzymes to discriminate between the common Sia linkages known to occur in sialoglycans (α2,3-, α2,6-, and α2,8-) or between the identity and arrangement of the underlying sugars has not been studied systematically.

In the current work we have analyzed the activity and specificity of the four mammalian neuraminidases against a panel of fluorescent 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY)-labeled) substrates containing the most common sialylated

oligosaccharides found in mammals: 3'SiaLacNAc (S1), 3'SiaLac (S2), $SiaLe_x$ (S3), $SiaLe_a$ (S4), $SiaLe_c$ (S5), 6'SiaLac (S6), and 6'SiaLacNAc (S7). These studies were combined with molecular modeling to define the structural basis of the enzyme specificities. Our data show for the first time that neuraminidases are capable of discriminating between different sialoglycans, providing crucial insight into their biological functions.

2.3 Experimental procedures

2.3.1 Synthesis of BODIPY-labeled sialyloligosaccharides

Sialyloligosaccharides containing ω-amino-spacers were synthesized as described previously (Pazynina, Tuzikov, Chinarev, Obukhova, & Bovin, 2002; Tuzikov, Gambaryan, Juneja, & Bovin, 2000) and labeled with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (succinimidyl ester) - BODIPY FLSE (Molecular Probes) (Mochalova et al., 2007). BODIPY-labeled sialyloligosaccharide neuraminidase substrates had the following structures: Neu5Acα2-3Galβ1-4GlcNAc-BODIPY (S1; $SiaLe_c$); Neu5Acα2-3Galβ1-3GlcNAc-BODIPY (S2; 3'SiaLac); Neu5Acα2-3Galβ1-4Glc-BODIPY (S3; $SiaLe_a$); Neu5Acα2-6Galβ1-4GlcNAc-BODIPY (S4; $SiaLe_x$); Neu5Acα2-6Galβ1-4Glc-BODIPY (S5; 6'SialLacNAc); Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc-sp-BODIPY (S6; 6'SiaLac); and Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc-sp-BODIPY (S7; 3'SiaLacNAc).

2.3.2 Neuraminidases

Neu1 was purified from mouse kidney tissue by affinity chromatography on a concanavalin A-Sepharose column followed by fast protein liquid chromatography gel filtration

on Superose-6 column, as previously described (Pshezhetsky & Potier, 1996). Neu2 and Neu3 were expressed in *E. coli* fused at N-terminal with maltose-binding protein and Neu4 as a glutathione S-transferase-fusion protein and purified as described (Albohy et al., 2010; Albohy et al., 2011; Y. Zhang et al., 2013). Neu2 and Neu4 carrying a streptavidin binding peptide tag at the C-terminus were also expressed in COS 7 cells and purified to electrophoretic homogeneity by affinity chromatography on Streptavidin agarose essentially as described (Dridi et al., 2013; Durand, Feldhammer, Bonneil, Thibault, & Pshezhetsky, 2010). Specific enzymatic activity of purified enzymes was measured against 4-Methylumbelliferyl α-D-*N*-acetylneuraminic acid (4MU-NANA) in 0.1 M Na-acetate buffer at the enzyme optimal pH (4.5 for Neu1, Neu3, and Neu4; and 5.5 for Neu2) and molecular mass was confirmed using FPLC size exclusion chromatography as described (Pshezhetsky & Potier, 1996). For the MS/MS analysis after separation by SDS-PAGE the gel pieces containing Neu2 and Neu4 were excised, and digested with trypsin (Promega). The tryptic peptides were analyzed by nano-liquid chromatography mass spectrometry (nanoLC-MS) using an Eksigent nano-LC LTQ-Orbitrap mass spectrometer system (Thermo Fisher Scientific) as described (Trost et al., 2009). Assignments of phosphorylation sites were validated through manual inspection of relevant MS/MS spectra.

2.3.3 Neuraminidase activity assay

Neuraminidase activity against BODIPY-labeled substrates was assayed as described by Mochalova et al. (Mochalova et al., 2007) with minor modifications. Briefly, the 15 µL of reaction mixture containing 8 mU (1 mU equals 1 nmol of 4MU-NANA substrate converted per h) of neuraminidase enzyme and a substrate at a final concentration of 20 µM, 10 µM, 5 µM, 2.5 µM,

1.2 µM or 0.6 µM in 0.1 M Na-acetate buffer, pH 4.5 for Neu1, Neu3, Neu4 or pH 5.5 for Neu2 was incubated at 37° C for 20 min in 96-well PCR plates (BioScience Inc.). For blank samples, neuraminidase was replaced with an equal volume of water. The reaction was terminated by the addition of 85 µL of ice-cold water and 10-µL aliquots of the reaction mixtures were applied to 96-well filter plates (Millipore, 40 µm Nylon Mesh) containing 40 µL of DEAE-Toyopearl 650M (Tosoh) resin per well. Prior to the assay, the resin was washed twice with 250 µL of water/well and the plates centrifuged at 50 *g* for 30 s to remove any excess water. After application of the reaction mixture, the DEAE-plates, were incubated for 1 min at room temperature and centrifuged at 50 *g* for 30 sec. Reaction products were eluted with two 95-µL aliquots of water by centrifugation of the plates at 50 *g* for 30 s. Combined eluent (200 µL) was transferred to 96-well ReaderBlack polystyrene plates (Life Science) and the amount of fluorescent product was measured using an EnVision 2104 Multilabel fluorimeter (Perkin Elmer) at emission wavelength of 535 nm and excitation wavelength of 485 nm. Three independent duplicate measurements were performed for each experimental condition. Kinetic parameters of enzymatic reactions were analyzed by non-linear regression using Prism Graphpad software.

2.3.4 Animals.

Mice with targeted disruption of the *neu*3 (*neu*3$^{-/-}$) and *neu*4 (*neu*4$^{-/-}$) genes and hypomorph mice with deficiency of CathA expression causing secondary 90% reduction of the Neu1 activity in tissues (*CathA*$^{S190A\text{-}Neo}$), all in C57BL/6NCrl genetic backgrounds have been previously described (Seyrantepe, Canuel, et al., 2008; Seyrantepe, Hinek, et al., 2008; Yamaguchi et al., 2012). Mice with a combined deficiency of Neu4 and Neu3 were obtained by intercrossing

Neu4 and *Neu3* knockout (KO) mouse strains. Doubly homozygous *Neu4*$^{-/-}$;*Neu3*$^{-/-}$ progeny were viable and their genotypes were confirmed by PCR of tail DNA. The absence of *Neu4* transcripts in total mRNA extracted from the brain of *Neu4*$^{-/-}$;*Neu3*$^{-/-}$ mice was confirmed by RT-PCR (Figure S1). Mice were housed in an enriched environment with continuous access to food and water, under constant temperature and humidity, on a 12 h light:dark cycle. Approval for the animal care and the use in the experiments was granted by the Animal Care and Use Committee of the Ste-Justine Hospital Research Center.

2.3.5 Neuraminidase assay in mouse brain tissues.

At the age of 12 weeks mice were sacrificed using CO_2 chamber and their brains extracted, snap-frozen with liquid nitrogen and kept at -80 ^{o}C. For measurement of neuraminidase activity 100 mg of frozen brain tissue was homogenized in water in a ratio of 100 mg of tissue per 300 µL of water in the 1.5 mL Eppendorf tubes using Kontes Pellet motorized pestle. Protein concentration in the homogenate was measured by Bradford method using the Bio-Rad reagent. The assay was performed as described above for recombinant neuraminidases but the reaction mixture contained an aliquot of homogenate corresponding to 150 µg of total protein and substrate in a final concentration of 10 µM. The reaction was carried on at 37 °C for 2 h after which it was terminated by the addition of 180 µL of ice-cold water. For blank samples, the reaction mixture contained buffer and substrate only but the same volume of homogenate was added after the termination of reaction with water.

2.3.6 Molecular modeling.

The structures of the substrates were generated using GLYCAM Web online utilities (*GLYCAM Web*, Complex Carbohydrate Research Center, University of Georgia, Athens, GA, http://www.glycam.com). A methyl group was used as the aglycon to simplify calculations. The substrates were docked in the active site of either a previously reported Neu3 homology model (Albohy et al., 2010) or a reported Neu2 crystal structure (Chavas et al., 2005). Docking calculations were performed with Autodock 4.0, and a grid box of 60*60*60 points centered at *C*2 of the Neu5Ac or 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid in the enzyme complex. Two hundred docked poses were generated and clustered, the lowest energy clusters maintaining key interactions (e.g. contact with the arginine triad and the *C*1 carboxylate) were selected for further study by molecular dynamics. Topology and coordinate files were generated for calculations in AMBER using the tLEaP module with solvation to generate a neutralized complex. An octahedral box of water (TIP3P) at 7 Å around the complex was used, with Na^+ ions added to neutralize the complex. Molecular dynamics simulations were performed using the AMBER 10 package. The GLYCAM06 force field was used with the carbohydrate substrates, and the AMBER ff99SB force field was used for the rest of the complex (Kirschner et al., 2008).

The complex was prepared for a production run of molecular dynamics by minimization and equilibration. The solvent molecules were first minimized while keeping the substrate constrained. This was followed by a minimization of the entire system. In both minimization steps, a steepest descent energy minimization was carried out for 50 cycles followed by 4950 cycles of conjugate gradient minimization. For equilibration, a total of 600 ps of annealing was used during which the temperature was increased every 50 ps from 5 to 300 K and then cooled

back from 300 to 5 K. This was followed by a short equilibration run of 200 ps during which the temperature of the system was gradually increased from 5 K to 300 K over 150 ps, followed by a constant temperature of 300 K for 50 ps. The production simulation (10 ns) was performed under constant pressure and temperature (NPT) with periodic boundaries. The temperature was kept at 300 K and the pressure at 1 atmosphere. The particle mesh Ewald (PME) method and SHAKE algorithm were used. Longer simulations (up to 20 ns) yielded substantially similar results. Simulations were analyzed for convergence by examining the total energy and potential energy of the run. Structures from the equilibrated phase of the production run were used for analysis.

2.4 Results

2.4.1 Substrate specificity of neuraminidases

Substrate specificity of the four mammalian neuraminidases was studied against a panel of synthetic sialo-oligosaccharides, typical for mammalian carbohydrate chains (Figure 1). The oligosaccharides differed in: (1) the type of linkage between Neu5Ac and Gal residues (α2,3 or α2,6; i.e. 3'SiaLac (S2) and 3'SiaLacNAc (S7) vs. 6'SiaLac (S6) and 6'SiaLacNAc (S5), respectively); (2) the presence or absence of the *N*-acetyl group at position two of the Glc residue (i.e. 3'SiaLacNAc (S7) vs. 3'SiaLac (S2)); (3) the linkage (1,4 or 1,3) between Gal and GlcNAc residues (i.e. 3'SiaLacNAc (S7) vs. $SiaLe_c$ (S1)); and (4) the presence or absence of a branching Fuc residue at GlcNAc (i.e. $SiaLe_a$ (S3) and $SiaLe_x$ (S4) vs. SiaLec (S1) and 3'SiaLacNAc (S7) respectively).

The activities against the above substrates were tested with recombinant human neuraminidases 2, 3 and 4 expressed in *E. coli* and purified as previously described(Albohy et al., 2010; Albohy et al., 2011; Y. Zhang et al., 2013). As we demonstrated previously, recombinant enzymes have specific activities against the fluorogenic substrate, 4MU-NANA and the pH optima similar to those of the enzymes purified from the mammalian tissues (Y. Zhang et al., 2013). However, it is possible that the mammalian neuraminidases expressed in bacteria, which lack post-translational modifications or an oligomeric structure characteristic for mammalian enzymes, may have different enzymatic properties. To test this, we also expressed Neu2 and Neu4 with a C-terminal TAP (SBP/CBP) tag in mammalian COS 7 cells and purified them using affinity chromatography on Streptavidin-agarose. Analysis of the purified enzyme by tandem mass spectrometry demonstrated that both Neu2 and Neu4 contained O-linked

phosphorylations and according to FPLC size exclusion chromatography Neu4 expressed in COS 7 cells formed homodimers with an apparent mass of ~120-kDa. Despite these differences with recombinant enzymes expressed in bacteria, we observed identical profiles of activities for the Neu2 and Neu4 proteins expressed in bacteria and mammalian cells against all substrates (data not shown). Based on these data, all further studies were performed using the recombinant enzymes.

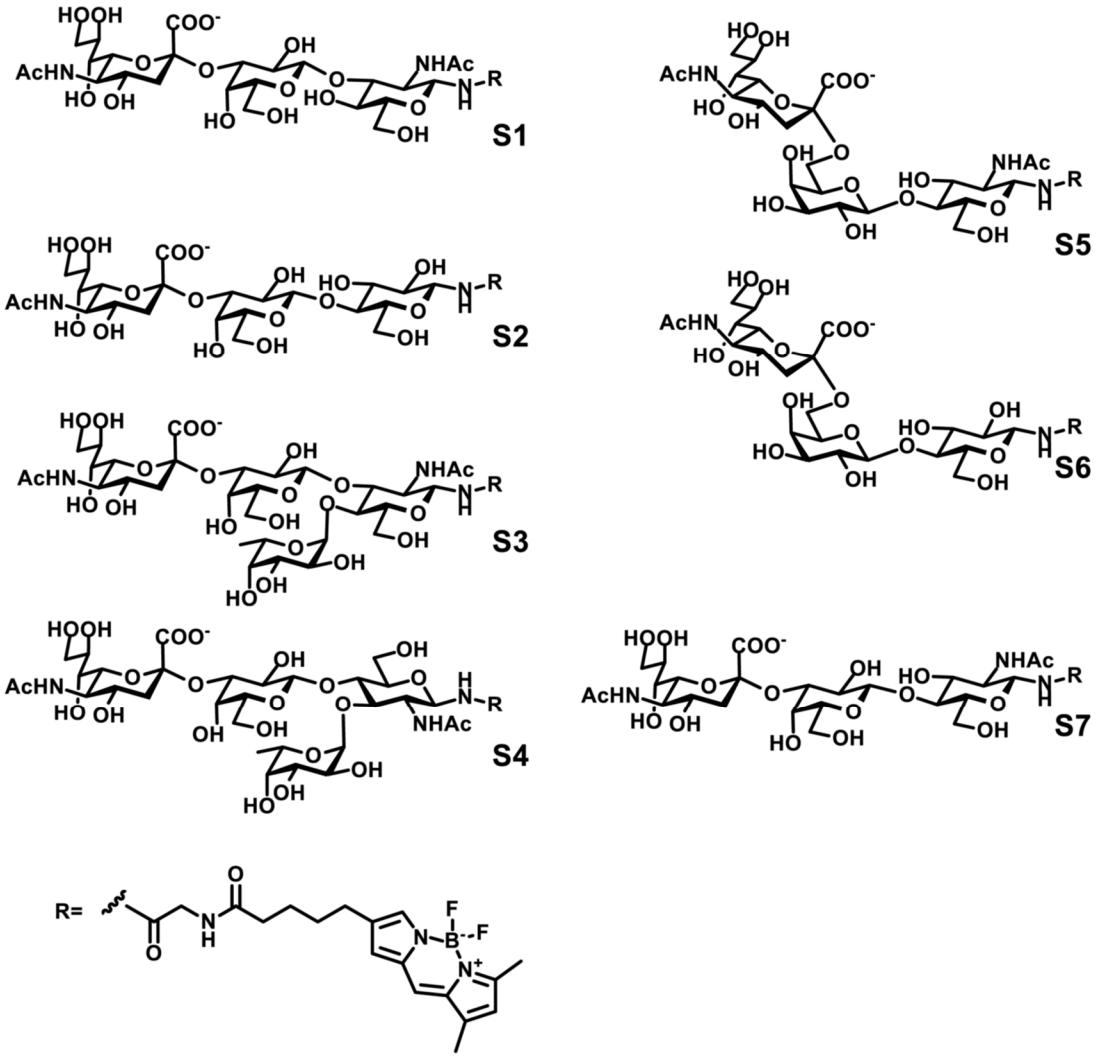

Figure 1. Chemical structures of the fluorescent substrates used in this study.

Since Neu1 is catalytically active only as a part of a multienzyme lysosomal complex with β-galactosidase and lysosomal carboxypeptidase, cathepsin A, we used the endogenous enzyme purified from mouse kidney tissue by affinity chromatography on concanavalin A-Sepharose followed by FPLC size exclusion chromatography as previously described (Pshezhetsky & Potier, 1996). Mouse and human Neu1 have a very high (83% identity, 89% similarity) amino acid homology and similar specificity (Pshezhetsky & Ashmarina, 2001).

For each enzyme the initial reaction rate was measured at a corresponding pH-optimum (4.75 for Neu1, Neu3 and Neu4 and 5.5 for Neu2) for six substrate concentrations between 0.6 and 20 μM. The same amount (8 mU) of the enzyme activity was added to the reaction mixture for each neuraminidase. After a 30-min incubation at 37 °C, the reaction was terminated by dilution with ice-cold water; the product was separated from unreacted substrate using ion-exchange chromatography on Toyoperl DEAE-650 and its concentration was measured using a spectrofluorimeter. In separate experiments we confirmed that under the conditions used the amount of liberated product was directly proportional both to the incubation time and to the amount of enzyme in the reaction mixture (data not shown). For each enzyme the dependence of the initial reaction rate on the substrate concentration could be described by the Michaelis-Menten equation allowing determination of K_M (Michaelis constant) and V_{max} (maximal velocity of enzymatic hydrolysis) values (Table 1). V_{max}/K_M ratios were used as a measure of specificity of neuraminidases towards corresponding the substrates (Figure 2).

In general, Neu3 and Neu4 were more active than Neu1 and Neu2 against most substrates in the panel. Importantly, neuraminidases also showed drastic differences in specificity towards substrates with 2,6-linked and 2,3-linked Sia. Neu3 showed similar activity for an α2,6-linked-

substrate, 6'SiaLac (S6), and the α2,3-linked isomer, 3'SiaLac (S2). The specificity of Neu1 was similar for the 6'SiaLacNAc (S5) and 3'SiaLacNAc (S7). Neu4 was capable of hydrolyzing α2,6-linked Sia substrates, but showed more than 3-fold-reduced specificity for 6'SiaLac (S6) and 6'SiaLacNAc (S5) as compared to 3'SiaLac (S2) and 3'SiaLacNAc (S7). Neu2 showed virtually no activity against substrates with α2,6-linked Sia (S5 and S6).

Table 1. Kinetic data from substrate studies with recombinant neuraminidases.

	S1	S2	S3	S4	S5	S6	S7
Neu1							
V_{max} (SE)	$3\cdot10^7$ ($4.4\cdot10^6$)	$2.8\cdot10^7$ ($2.4\cdot10^6$)	$5\cdot10^{25}$ ($6.9\cdot10^{24}$)	$2.4\cdot10^7$ ($9.9\cdot10^6$)	$1.4\cdot10^7$ ($1.8\cdot10^6$)	$2\cdot10^7$ ($4.2\cdot10^6$)	$3.2\cdot10^7$ ($4.5\cdot10^6$)
K_M (SE)	0.31 (0.07)	0.24 (0.04)	$1.3\cdot10^{18}$ ($1.3\cdot10^{17}$)	0.42 (0.26)	0.27 (0.06)	0.50 (0.14)	0.39 (0.08)
V_{max}/ K_M	$9.7\cdot10^7$	$1.2\cdot10^8$	$3.8\cdot10^7$	$5.7\cdot10^7$	$5.2\cdot10^7$	$4\cdot10^7$	$8.2\cdot10^7$
Neu2							
V_{max} (SE)	$1.7\cdot10^7$ ($1.2\cdot10^6$)	$2.5\cdot10^7$ ($3.7\cdot10^6$)	$1\cdot10^{26}$ ($1.5\cdot10^{25}$)	$2.7\cdot10^7$ ($2.3\cdot10^6$)	$9.9\cdot10^{20}$ ($1.3\cdot10^{20}$)	$9.3\cdot10^{23}$ ($7.3\cdot10^{23}$)	$2\cdot10^7$ ($9.2\cdot10^5$)
K_M (SE)	0.14 (0.02)	0.38 (0.08)	$5\cdot10^{18}$ ($6.2\cdot10^{17}$)	0.42 (0.05)	$1.5\cdot10^{14}$ ($2.5\cdot10^{13}$)	$8.9\cdot10^{16}$ ($1.2\cdot10^{16}$)	0.23 (0.02)
V_{max}/ K_M	$1.2\cdot10^8$	$6.6\cdot10^7$	$2\cdot10^7$	$6.4\cdot10^7$	$6.6\cdot10^6$	$1\cdot10^7$	$8.7\cdot10^7$
Neu3							
V_{max} (SE)	$2\cdot10^7$ ($1.7\cdot10^6$)	$2.7\cdot10^7$ ($2.9\cdot10^6$)	$4.4\cdot10^7$ ($4.2\cdot10^6$)	$2.3\cdot10^7$ ($2.6\cdot10^6$)	$2.8\cdot10^7$ ($3\cdot10^6$)	$2.2\cdot10^7$ ($2.2\cdot10^6$)	$1.6\cdot10^7$ ($1.6\cdot10^6$)
K_M (SE)	0.06 (0.01)	0.14 (0.03)	0.51 (0.07)	0.13 (0.03)	0.23 (0.04)	0.13 (0.03)	0.06 (0.02)
V_{max}/ K_M	$3.3\cdot10^8$	$1.9\cdot10^8$	$8.6\cdot10^7$	$1.8\cdot10^7$	$1.0\cdot10^8$	$1.7\cdot10^8$	$2.7\cdot10^8$
Neu4							
V_{max} (SE)	$1.6\cdot10^7$ (1.4·106)	$3\cdot10^7$ $(5.4\cdot10)^6$	$4.5\cdot10^7$ $(1.7\cdot10)^7$	$4.4\cdot10^7$ ($7.4\cdot10^6$)	$5.2\cdot10^6$ ($1.3\cdot10^6$)	$1.1\cdot10^7$ ($3.6\cdot10^6$)	$1.9\cdot10^7$ ($2.8\cdot10^6$)
K_M (SE)	0.05 (0.01)	0.20 (0.07)	0.85 (0.42)	1.18 (0.83)	0.12 (0.06)	0.24 (0.14)	0.11 (0.04)
V_{max}/ K_M	$3.2\cdot10^8$	$1.5\cdot10^8$	$5.3\cdot10^7$	$3.7\cdot10^7$	$4.3\cdot10^7$	$4.6\cdot10^7$	$1.7\cdot10^8$

Values shown are means and standard errors of three independent experiments

The nature of the residue at the reducing end (Glc or GlcNAc) had only a minor effect on the activity of all neuraminidases, with a similar specificity observed for 3'SiaLac (S2) and

3'SiaLacNAc (S7) and for 6'SiaLac (S6) and 6'SiaLacNAc (S5). The central Gal-GlcNAc linkage (β1,3 or β1,4) had a noticeable effect on Neu4 activity, which showed 2-fold-reduced specificity for the β1,4-linked GlcNAc substrate (S1 vs. S7).

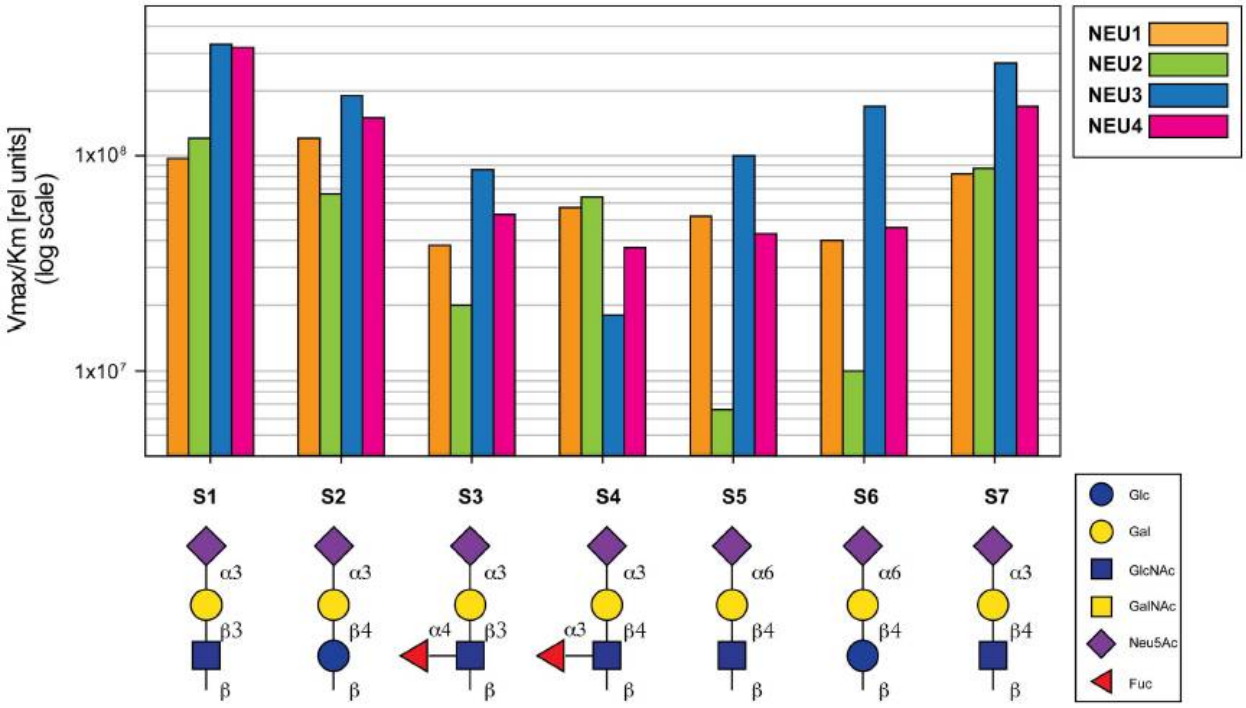

Figure 2. Substrate specificity of mammalian neuraminidases.
Vmax/KM values are plotted on a logarithmic scale. Values shown are means of three independent experiments.

The branching Fuc residue generally reduced substrate activity for all neuraminidases. This effect was more pronounced when Fuc was attached in α1,4 position to GlcNAc residue (S3 vs. S1) rather than in α1,3 position (S4 vs. S7). Of all enzymes Neu4 was the most affected by the branching Fuc, which reduced the specificity for the corresponding substrates as much as ~6-fold. Neu1 was least affected by fucosylation, with only 2-fold reduction of specificity for branching Fuc attached both in α1,4 and α1,3 positions.

2.4.2 Molecular modeling of substrate binding in the active sites of Neu2 and Neu3.

To interpret the differences in the specificities of the enzymes we used molecular modeling to dock specific substrates to the active site of interest. There are limited structural data available for the mammalian neuraminidases with structures reported only for Neu2. Although homology models of the remaining three isoenzymes have been described, only Neu3 has been experimentally tested (Albohy et al., 2010). We restricted our modeling studies to the Neu2 and Neu3 active sites for which we identified sets of substrates with large differences in the V_{max}/K_m ratio.

Table 2. Predicted protein-ligand contacts in Neu2 and Neu3

		S1 Neu2	S5 Neu2	S1 Neu3
monosaccharide	site	residue	residue	residue
Neu5Ac	C1-COOH	R304 R237 Y334	R304 R21	R340 R25 Y370
	4-OH	R41	R41	R45
	5-NH	Y334		E225 Y370
	5-C(O)	R41		R45
	7-OH	E111		R245
	8-OH	E111	E111	D50
	9-OH	R237	E218 R237	Y179
Gal	2-OH		K44*	
	3-OH		Y359	
	4-OH			
	6-OH			
GlcNAc	2-NH			
	3-OH		Q112	
	4-OH	E111		R245
	5-O			
	6-OH	D46Q112	D46	

*Backbone interactions (carbonyl or amide).

Models of enzyme-substrate complexes were generated using molecular dynamics. Initial protein structures were based on crystallographic data in the case of Neu2 (Chavas et al., 2005), and a homology model in the case of Neu3 (Albohy et al., 2010). Substrates were first docked

into the active site using Autodock 4, and then subjected to molecular dynamics to convergence, followed by minimization as described in Materials and Methods (Table 2).

Neu2 showed the largest differences in activity against substrates S1 ($SiaLe_c$) and S5 (6'SiaLacNAc). The two substrates are isomers differing in the two glycosidic linkages of Sia-Gal (α2,3 vs. α2,6) and the Gal-GlcNAc (β1,3 vs. β1,4) residues. The model of S1 binding to the active site of Neu2 includes most of the expected key interactions seen in co-crystals with sialosides (Figure 3) (Chavas et al., 2005), including binding of the *C*1-carboxylate of Sia with the Arg triad. The GlcNAc residue B face is placed on the top of the hydrophobic side chain of Glu112 forming favourable non-polar contacts. Thus, the α2,3 glycosidic linkage between Sia and Gal residues allows the S1 substrate to form the stable complex with the active site of the enzyme. In contrast, the α2,6 glycosidic linkage of S5 makes it difficult for the substrate to fit into the Neu2 active site without adopting an unfavorable conformation. The best orientation of S5 in the Neu2 active site results in Sia adopting a twist-boat conformation in order to preserve interactions of the Arg triad and the *C*1-carboxylate. The GlcNAc A face of S5 develops an interaction with a hydrophobic patch of the Neu2 surface (C_b, D46). This model suggests that the reduced activity of S6 may also be a result of the α2,6 linkage forcing an unfavorable orientation of the Sia residue. Considering that S7, which also contains a β1,4 glycosidic linkage to GlcNAc, has activity comparable to S1 with Neu2; we conclude that the glycosidic linkage of Sia is the major determinant of substrate activity in this case.

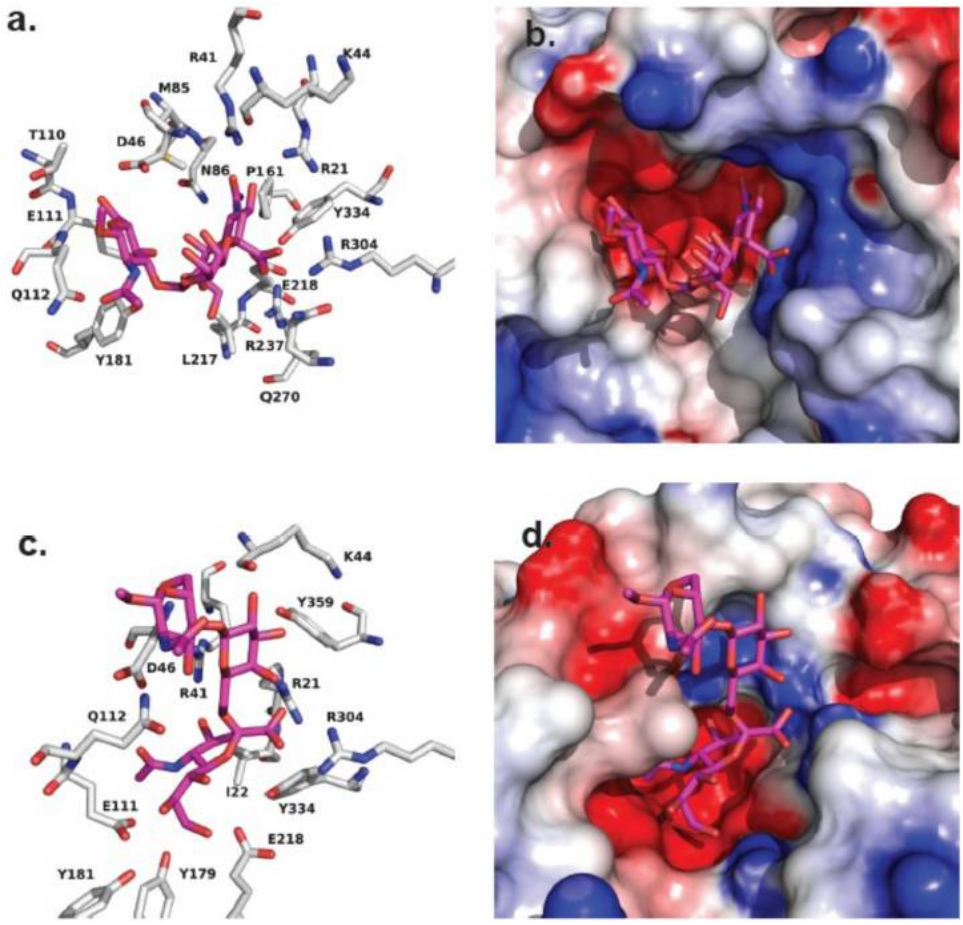

Figure 3. Modeling of S1 and S5 binding to the Neu2 active site.

(a) The substrate, S1, binds to the active site with expected contacts to the arginine triad and glycerol side chain. The GlcNAc residue B face is placed on the top of the hydrophobic side chain of Q112, forming a favorable interaction. The α2,3-glycosidic linkage between Neu5Ac and Gal allows S1 to form a relatively stable complex. (b) The same position for S1 as shown in (a), but with a surface representation of the Neu2 active site. (c) The α2,6-glycosidic linkage between the Neu5Ac and Gal residues in S5 makes it difficult for the substrate to fit into the active site without adopting an unfavorable conformation. The Neu5Ac residue adopts a twist boat conformation to preserve interactions with the Arg triad. The GlcNAc A face is interacting with a hydrophobic patch in the Neu2 active site, which is likely unfavorable. (d) The same pose for S5 as shown in (c), but with a surface representation of the Neu2 active site.

We next examined the interaction of the most active (S1; $SiaLe_c$) and least active (S3; $SiaLe_a$) substrates with the active site of Neu3. The only structural difference between the two substrates is the branching Fuc1,4 residue of S3. A model of S1 binding to the active site of Neu3 was generated using the procedure described in Materials and Methods (Figure 4). In this model, the Neu5Ac residue adopts a twist boat conformation in order to preserve many key interactions

(Varghese, McKimmbreschkin, Caldwell, Kortt, & Colman, 1992). However, when we attempted the same procedure for S3, we were unable to identify any suitable orientation for the substrate that would place the Sia residue in the correct orientation. Upon inspection of the S1-Neu3 model, we concluded that the branching fucosyl residue of S3 would be directed deeper into the binding site where it would clash with protein side chains (R245 and E225). Thus, we concluded that branching at *O*4 of GlcNAc prevented appropriate binding of the substrate. This would likely be less of an issue for compounds like S4, which could accommodate the α1,4-fucosyl residue by projection into solvent when the Gal moiety is linked in β1,3 position.

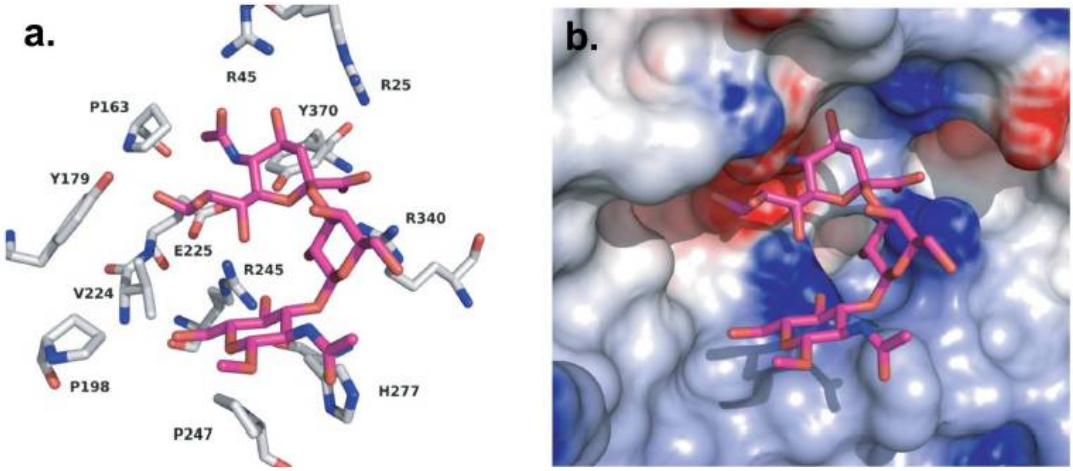

Figure 4. Modeling of S1 binding to Neu3 active site.
(a) Interactions of S1 with amino acid residues in the active site of the Neu3 homology model. (b) The same position of S1 shown in (a), with a surface representation of the active site of Neu3.

2.4.3 Neuraminidase activity in the mouse brain tissues.

In order to determine if the detected differences in the specificities of neuraminidases against the BODIPY-labeled sialylated oligosaccharides could be used to discriminate between the enzymes *in vivo*, we have assayed neuraminidase activity in the tissues of available previously

described gene-targeted mouse models deficient in Neu1 (*CathA*$^{S190A\text{-}Neo}$, *Neu1 KI*), Neu3 (*Neu3*$^{-/-}$) and Neu4 (*Neu4*$^{-/-}$)(Seyrantepe, Canuel, et al., 2008) (Seyrantepe, Hinek, et al., 2008; Yamaguchi et al., 2012). As previous reports have suggested that Neu3 and Neu4 have similar substrate specificity (Seyrantepe et al., 2004; Yamaguchi et al., 2005) we also produced a mouse line with a double Neu3/Neu4 deficiency (*Neu3*$^{-/-}$*;Neu4*$^{-/-}$) by cross-breeding individual knockouts. As expected the expression levels of both *Neu3* and *Neu4* were below the detection limit in the tissues of the *Neu3*$^{-/-}$*;Neu4*$^{-/-}$ mice (Figure S1).

We assayed the neuraminidase activity in the mouse brain tissues, where approximately equal amounts of Neu1, Neu3 and Neu4 (and only negligible amount of Neu2) were previously found in the WT mice(Koseki et al., 2012). Acidic neuraminidase activity, assayed using 4MU-NaNa as substrate, was reduced to 38% of the WT level in the brain tissues of *Neu1 KI* mouse, 64% in the *Neu3*$^{-/-}$ mouse, 59% in the *Neu4*$^{-/-}$ mouse, and 36% in the *Neu3*$^{-/-}$*;Neu4*$^{-/-}$ mouse (Figure S2), indicating that each of the three neuraminidases (Neu1, Neu3 and Neu4) contribute approximately 30% of the net brain neuraminidase activity against 4MU-NaNa. In contrast, the residual neuraminidase activity levels measured in the brain tissues of Neu-deficient mice against BODIPY-labeled sialylated oligosaccharides were drastically different between substrates (Figure 5). First, we did not detect a significant reduction of the activity for any substrate except of the substrate S4 ($SiaLe_x$) in the tissues of Neu1-deficient mice as compared to that in the WT mice. This is not surprising because S4 is the only substrate for which Neu1 showed higher specificity then Neu3 or Neu4 (Figure 2), whereas the rest of the substrates were hydrolyzed by Neu3 and Neu4 with the rates at least two-fold higher than that of Neu1. Second, the activity in the tissues

of both *Neu3* and *Neu4* KO mice against the substrates S1, S4, and S7 (equally specific in vitro for Neu3 and Neu4) was significantly lower than that in the tissues of WT mice (Figure 5).

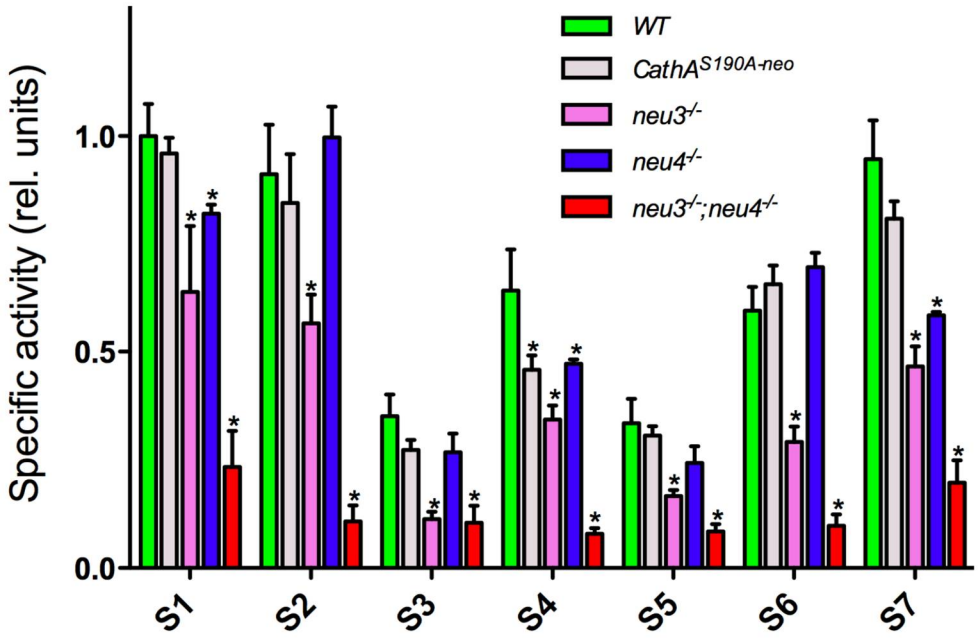

Figure 5. Neuraminidase activity in mouse brain tissues.
The activity was measured in the homogenates of whole brain tissues of wild-type C57Bl6 mice (*WT*) and gene-targeted C57Bl6 mice deficient in Neu3 (*Neu3*$^{-/-}$), Neu4 (*Neu4*$^{-/-}$), Neu1 (*CathA*$^{S190A\text{-}neo}$) and double-knockout *Neu3*$^{-/-}$;*Neu4*$^{-/-}$ mice against BODIPY-labeled sialylated oligosaccharides in 10 µM concentration. Values are shown as means (±S.E). N-value for each genotype is as follows: for WT and *Neu3*$^{-/-}$;*Neu4*$^{-/-}$ n=8, for *Neu3*$^{-/-}$ and *Neu4*$^{-/-}$ n=6; for *CathA*$^{S190A\text{-}neo}$ n=4. * -significantly different from WT ($P<0.05$) by repeated measurements ANOVA.

This was consistent with the data on the residual activity against S1, S4, and S7 in double-knockout *Neu3*$^{-/-}$;*Neu4*$^{-/-}$ mice which was significantly lower than that in *Neu3* or *Neu4* knockouts. On the other hand, the activity measured against the Neu3-specific substrates S3, S5 and S6 was similar in the tissues of *Neu4* KO and WT mice but was equally reduced in the tissues of *Neu3* KO and double-KO *Neu3*$^{-/-}$;*Neu4*$^{-/-}$ mice. These substrates can be potentially used for specific measurements of Neu3 activity in tissue homogenates. Finally, although *in vitro* Neu3 and Neu4 were equally specific for S2 the activity against this substrate in the tissues of *Neu4* knockout

mice was not reduced as compared with WT mice. In the *Neu3* KO mice the activity measured against S2 was lower than in WT but higher than in the double-KO *Neu3*$^{-/-}$;*Neu4*$^{-/-}$ mice, which may imply that changes in the *Neu3* expression may help to compensate for the deletion of the *Neu4* gene product, consistent with a significant increase of the *Neu3* mRNA in the brain tissues of *Neu4*$^{-/-}$ as compared to that in WT mice (Figure S1).

2.5 Discussion

Phylogenetic analysis of the sialidase/neuraminidase family suggests that it has originated in bacteria, and then specialized forms were developed in higher organisms (Giacopuzzi, Bresciani, Schauer, Monti, & Borsani, 2012). Neu1 was likely the first to form, further evolving into Neu2 and a common precursor of Neu3 and Neu4. The development of members of the neuraminidase family other than Neu1 was most likely driven by a classical mechanism of subfunctionalization, where different isoforms would have distinct biological functions and substrate specificity (Giacopuzzi et al., 2012).

Our analysis shows that Neu1, the oldest member of the neuraminidase family, also has the broadest specificity. The Neu1 enzyme is active against both α2,3 and α2,6 linked Sia. Its activity is not affected by the core type (1,3 or 1,4 bond between Gal and GlcNAc residues) and is only slightly inhibited by the branching Fuc residues. Such broad substrate specificity explains the wide diversity of the biological substrates and functions of Neu1. First, as a lysosomal enzyme Neu1 catabolizes a wide range of glycan chains on sialylated glycoproteins, which contain both α2,3 and α2,6 linked Sia and are often fucosylated (Pshezhetsky & Ashmarina, 2001). The role of Neu1 in the lysosomal catabolism of gangliosides is still disputed. The enzyme has much lower in

vitro activity against different gangliosides as compared with Neu3 and Neu4, which could be compensated by the high intra-lysosomal content of Neu1. The data on storage of gangliosides in human sialidosis patients have been controversial [reviewed in (Pshezhetsky & Ashmarina, 2001)]. Our current data show that Neu1 is capable of cleaving Sia residues from glycans containing both GlcNAc and Glc in the third position, and present in di-sialo and mono-sialo gangliosides, respectively. Besides catabolism, Neu1 has important regulatory functions, such as desialylation of multiple surface receptors in immune, metabolic, and cell proliferation pathways [reviewed in (Pshezhetsky & Hinek, 2011)]. The broad specificity of Neu1 we report here helps to explain the enzyme's ability to act on a wide range of receptors with diverse structures of the glycan chains.

Of all neuraminidases, Neu2 has the narrowest specificity. Interestingly, the enzyme also has a very restricted expression pattern: it was cloned from skeletal muscle (Miyagi et al., 1993; Monti, Preti, Rossi, et al., 1999) and thymus (Kotani et al., 2001) tissues, but placenta, testis, ovary and lungs have been recently identified as its major expression sites in humans. The enzyme has been proposed to cleave the G_{M3} ganglioside in differentiating myoblasts (Fanzani et al., 2003; Sato & Miyagi, 1996), but no direct evidence has been reported. In PC-3 prostate cancer and melanoma cells, Neu2 activity has been correlated with invasive and metastastatic potential (Koseki et al., 2012); however, the biological substrates of Neu2 involved in this process remain to be identified. Our data confirm previous observations that Neu2 has a preference for 2,3-linked Sia(Khedri et al., 2012) but also show that Neu2 is active on glycan chains lacking a branching Fuc residue.

The Neu3 and Neu4 isoenzymes show highest phylogenetic similarity of all mammalian neuraminidases (Giacopuzzi et al., 2012), and have relatively similar profiles of substrate specificity. Both enzymes have higher activity when compared to Neu1 and Neu2 against all substrates from our panel with an exception of S4. This observation could be the result of the fact that all substrates contain a bulky hydrophobic aglycone (BODIPY). Previous studies have found that Neu3 prefers substrates containing a hydrophobic aglycone (Sandbhor et al., 2011) presumably interacting with a hydrophobic binding site in Neu3 containing V222, V224, P198, P247, I117, and V118 residues of the enzyme (A. Albohy, M.R. Richards, and C.W. Cairo, unpublished). Additionally, both Neu3 and Neu4 are known to cleave ganglioside substrates in vitro, suggesting that gangliosides are their main physiological substrates (Hasegawa et al., 2007; Seyrantepe et al., 2004; Y. Wang et al., 2001). For both Neu3 and Neu4 the most active substrate is S1 containing α2,3 linked Sia and β1,3 linked GlcNAc at the third position, followed by S7 containing β1,3 linked GlcNAc. Neu4 has previously been shown to cleave $SiaLe_x$ and $SiaLe_a$ structures, and our data confirm that the equivalent glycans (S4 and S3) are substrates of this enzyme (Shiozaki, Yamaguchi, Takahashi, Moriya, & Miyagi, 2011b). The only apparent difference in specificity between Neu3 and Neu4 is their ability to cleave α2,6 linked Sia. While Neu4 showed reduced activity towards substrates containing α2,6 linked Sia, Neu3 showed only a slight reduction. Similarly, the activity of Neu3 was less affected by branching Fuc residues. Since α2,6 linked Sia are found preferentially in glycans of glycoproteins, we speculate that membrane glycoproteins may be among the physiological substrates of Neu3, which is in contrast to the common notion that Neu3 is a purely ganglioside-specific neuraminidase.

In conclusion, this study is the first systematic analysis of the substrate specificity of mammalian neuraminidases at the level of glycan structures. Our data reveal significant differences in the substrate specificity between the four mammalian neuraminidases presumably resulting from the structural organization of their active sites and suggest that presence of four different genes encoding enzymes responsible for removal of Sia residues from glycoconjugates is explained by the need for different substrate specificities. The described substrate preferences for each neuraminidase may be crucial therefore for future identification of their biological targets and pathways involving these enzymes. Besides, our results on measurement of neuraminidase activity in mouse models deficient in individual neuraminidases show that some of described substrates or their analogues can be potentially used to measure activities of the specific sialidases *in vivo*.

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2.6 Supporting Information

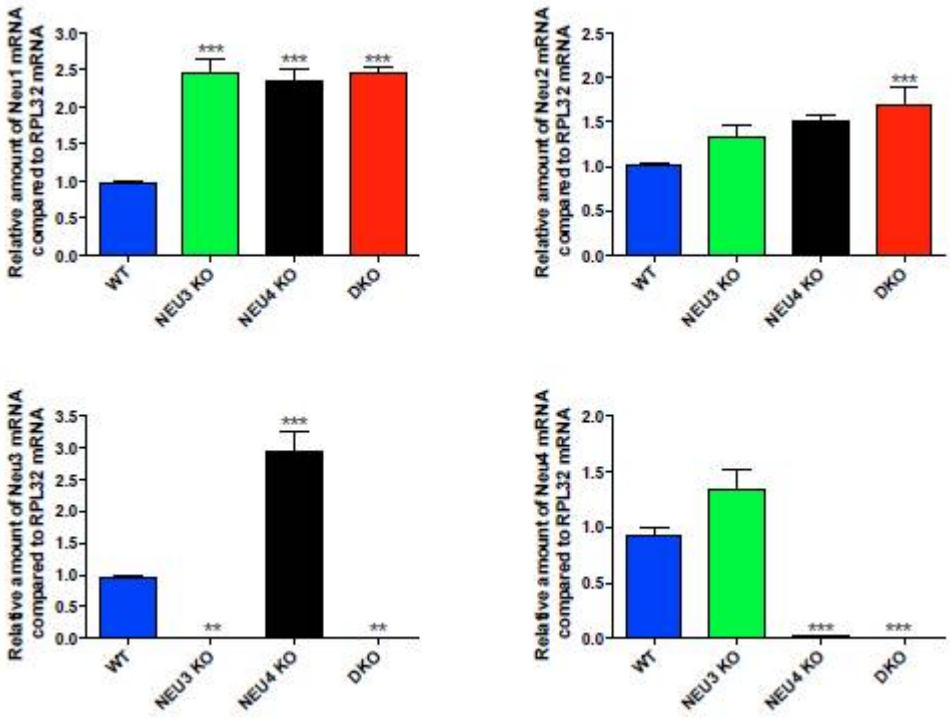


Figure S1. Relative expression of neuraminidase mRNA in mouse brain tissues.
Total mRNA was extracted from whole brains of 16 week-old WT, *Neu3*$^{-/-}$, *Neu4*$^{-/-}$ and double-knockout *Neu3*$^{-/-}$;*Neu4*$^{-/-}$ mice and analyzed for *Neu1, Neu2*, *Neu3* and *Neu4* expression by qRT-PCR The values were corrected for the level of control *RPL32* mRNA. ** and *** -significantly different from WT ($P<0.01$ and $P<0.001$, respectively) by repeated measurements ANOVA.

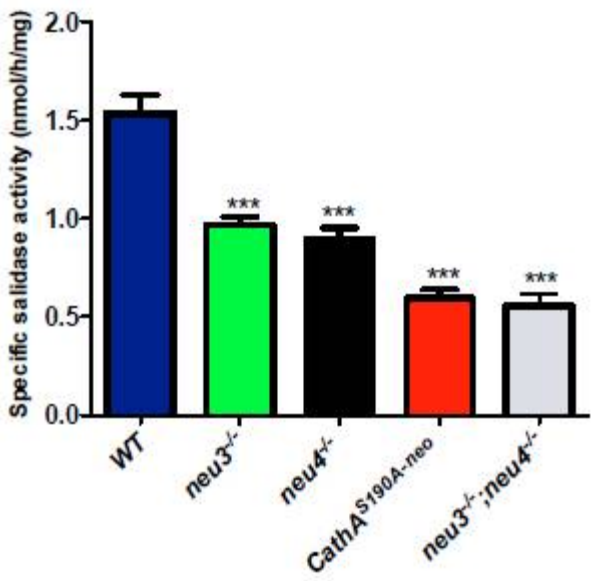


Figure S2. 4MU-NANA neuraminidase activity in mouse brain tissues.

Brain tissues of 16 week-old WT, *Neu3*$^{-/-}$, *Neu4*$^{-/-}$, *CathA*$^{S190A\text{-}neo}$ (*Neu1 KI*) and double-knockout *Neu3*$^{-/-}$;*Neu4*$^{-/-}$ mice and analyzed for neuraminidase activity against 4MU-NANA. Values are shown as means (±S.E). N-value for each genotype is as follows: WT and *Neu3*$^{-/-}$; *Neu4*$^{-/-}$ n=8, *Neu3*$^{-/-}$ and *Neu4*$^{-/-}$ n=6; *Neu1 KI* n=4. *** -significantly different from WT (P<0.001) by repeated measurements ANOVA.

Chapter 3. Neuraminidases 1 and 3 as triggers for atherosclerosis

(Paper in preparation)

3.1 Abstract

Atherosclerosis is a chronic vascular disease characterized by lipid retention and inflammation of the vessel wall. The disease starts from the uptake by resident macrophages of atherogenic modified low-density lipoproteins (LDL) resulting in formation of arterial fatty streaks and eventually atheromatous plaques. Increased plasma sialic acid levels or reduced sialylation of LDL and aortic endothelium have been associated previously with atherosclerosis and coronary artery disease in human patients but the mechanism underlying this association has never been explored. In the current study, we investigated the hypothesis that neuraminidases present on the surface of hematopoietic cells and/or arterial endothelium contribute to the development of atherosclerosis by removing sialic acid residues from the glycan chains of LDL glycoproteins and glycolipids. Our results demonstrate that in vitro desialylation of a major LDL glycoprotein – Apolipoprotein B 100 (ApoB) by human neuraminidases 1 and 3 increases the uptake of human LDL by cultured human macrophages, but not by hepatocytes. It also leads to increased accumulation of LDL in the aortic wall of mice. We further show that in the murine model of atherosclerosis, Apolipoprotein E (*ApoE*) knockout mice, genetic deficiency of neuraminidases 1 and 3 significantly delays the formation of fatty streaks in the aortic root without affecting the plasma cholesterol and LDL levels. Together, our results suggest that neuraminidases 1 and 3 trigger the initial phase of atherosclerosis, and formation of aortic fatty streaks by reducing sialylation of LDL and increasing their uptake rate.

3.2 Introduction

Atherosclerosis, a chronic inflammatory disease of the medium and large arteries, is currently the most common cause of heart attacks, strokes and vascular disease (Lozano et al., 2012). The disease manifests with endothelial disruption, an inflammatory cascade, proliferation of smooth muscle cells, migration of monocytes into the tunica media and formation of atheromatous plaques (Spitz et al., 2016) occurring initially at sites of reduced blood flow. Previous studies have identified a vast number of risk factors contributing to atherosclerosis in the human population including hyperlipidemia, smoking, hypertension, genetic predisposition, age, sex and obesity (Kalanuria, Nyquist, & Ling, 2012), however the cellular, biochemical and molecular mechanisms underlying plaque development are still not fully understood. A critical role in the initiation and progression of atherosclerosis belongs to the activation of endothelial cells (Meager, 1999). It leads to secretion of proinflammatory cytokines, chemokines and increased expression of the adhesion surface molecules, which results in leukocyte adhesion and migration into the subendothelial space, where they differentiate into macrophages (Weber, Zernecke, & Libby, 2008). Another crucial step in atherogenesis is infiltration of low-density lipoproteins (LDL) from the circulation into the subendothelial space of the artery wall, where they become modified and recognized by residential macrophages (Lusis, 2000). The uptake of modified LDL by macrophages leads to uncontrolled accumulation of cholesterol converting them to foam cells and triggers a cascade of immune responses that collectively lead to atheroma (X. H. Yu et al., 2013).

A high level of circulating cholesterol associated with LDL particles is a well-known risk factor for the development and progression of atherosclerosis (Bentzon et al., 2014; Keys, 1997; Martin et al., 1986). However at least 46% of first cardiovascular events occur in people with LDL levels at the normal range (Packard & Libby, 2008), suggesting that atherosclerosis is triggered not only by the increase of LDL level but also by changes in their composition including chemical modification of LDL molecules (Ahotupa et al., 2010). Oxidation or acetylation of LDL lipids in vitro makes them ligands of scavenger receptors on the surface of macrophages and increases their uptake, but it is still unclear whether oxidation is also the primary atherogenic LDL modification *in vivo* or other types of modifications play an equally or even more important role. One of such modifications potentially leading to atherosclerosis is desialylation: removal of terminal Sia from the glycan chains associated with surface LDL glycoproteins and glycolipids (mainly G_{M2} ganglioside). Glycosylation plays an important role in the biology of LDL and all LDL particles contain sialylated oligosaccharides (Millar, 2001; Orekhov et al., 1991). Specifically, ApoB, the core protein in human plasma LDL contains from 12 to 14 sialic acid residues as a part of its N-linked glycans (Millar, 2001). In contrast to oxidation, desialylation is a naturally occurring modification of LDL. It has been shown that the Sia content of LDL in patients with atherosclerosis is lower than of that in healthy subjects (Ruelland et al., 1993; Tertov et al., 1993). Desialylated LDL are rapidly taken up and accumulated by peripheral blood monocytes and smooth muscle cells isolated from human arterial intima via a mechanism involving surface lectin receptors (Bartlett et al., 2000). Desialylation of LDL causes changes in the structure of ApoB and results in accumulation of neutral lipids and cholesteryl esters in human aortic intimal cells (Tertov et al., 1992).

Neuraminidases (encoded in humans by the *NEU1-NEU4* genes) catalyze the removal of sialic acids from glycoproteins, oligosaccharides, and sialylated glycolipids (Pilatte, Bignon, & Lambre, 1993). Neuraminidases 1-4 have different, yet overlapping tissue expression, intracellular localization and substrate specificity. They play important physiological roles, regulating the immune response, cell proliferation, metabolism, normal development and carcinogenesis by desialylation of a wide spectrum of physiological substrates (Pshezhetsky & Ashmarina, 2013). In the current work, we demonstrate that neuraminidases 1 and 3 are also involved in the early stage of atherosclerosis development, suggesting that they may become a therapeutic target for preventing or treatment of this disease.

3.3 Materials and methods

3.3.1 Isolation of LDL and LPDS

Low density lipoproteins (LDL) (1.020 < d < 1.063 g/ml) were isolated from EDTA-anticoagulated blood plasma obtained from healthy normolipidemic human donors by sequential ultracentrifugation as described (Levy et al., 1990). The isolated LDL fraction was dialyzed against phosphate-buffered saline (PBS) containing 1 mM EDTA at 4^{o}C. Lipoprotein-deficient serum (LPDS) was prepared by ultracentrifugation at a density of 1.25 g/ml (Levy et al., 1990) and dialyzed against PBS at 4^{o}C. Protein concentration was measured using a Bio-Rad Bradford reagent.

3.3.2 LDL modification and labeling

Isolated LDL were desialylated by overnight treatment at 37°C with human recombinant neuraminidases (Smutova et al., 2014) in a ratio of 1 mU of enzyme per 10 μg of LDL. Before adding the enzyme, the pH of the LDL fraction was adjusted to the pH optimal for the corresponding neuraminidase (4.5 for Neu1, Neu3, and Neu4; and 5.5 for Neu2). Desialylation of LDL was confirmed by lectin blot as described below. To prepare oxidized LDL the sample was dialyzed against PBS supplemented with 5 μmol/L $CuSO_4$ and incubated at 37°C for 24 hours. All LDL fractions were used within 2 weeks after isolation and filtered (0.22 μm pore size) before each experiment.

Native and modified LDL were labeled with a fluorescent 3,3'-dioctadecylindocarbocyanine (DiI) dye (Molecular Probes) essentially as described (Pitas, Innerarity, Weinstein, & Mahley, 1981) with minor changes. Briefly, DiI dissolved in dimethyl sulfoxide (DMSO) at a concentration of 3 mg/ml was added to 2 ml of LPDS containing 1 mg of LDL to a final concentration of 300 μg of DiI per 1 mg of LDL protein. The sample was then incubated at 37 °C for 24 h in the dark. Then, the density of the incubation mixture was increased to 1.063 g/ml by adding KBr and the sample was subjected to ultracentrifugation to re-isolate LDL. Labeling of native or modified LDL with Alexa Fluor 488 (Invitrogen) was performed following the manufacture's protocol. After the labeling all LDL fractions were dialyzed against PBS.

3.3.3 Production and purification of human neuraminidases 1-4

Neu1 was purified from mouse kidney by affinity chromatography on a concanavalin A-Sepharose column followed by fast protein liquid chromatography gel filtration on Superose-6

column, as previously described (Pshezhetsky & Potier, 1996) or expressed as a His-tagged protein in HEK293 in a crude lysate (Pshezhetsky & Potier, 1996). Neu3 and Neu2 were expressed as N-terminal MBP fusion proteins in E. coli and purified as previously reported (Albohy et al., 2010). Neu4 was expressed as a GST fusion protein in E. coli and purified as previously reported (Albohy et al., 2011). Inhibition assays cleavage was performed using 4-methylumbelliferyl α-D-N-acetylneuraminic acid (4MU-NANA) and GM3 as substrates as reported previously (Y. Zhang et al., 2013). In vitro assays were conducted in 0.1 M sodium acetate buffer at optimum pH for each enzyme (4.5 for Neu1, Neu3 and Neu4; 5.5 for Neu2) (Y. Zhang et al., 2013). In a typical experiment the reaction mixture contained 50 µl of 0.1 acetate buffer, 25 µl of 4MU-NANA substrate 0.75 mM solution and 25 µl of the enzyme solution typically containing 0.2-0.5 µg of protein. Protein concentration was assayed according to Bradford (Bradford, 1976). After 30 min incubation at 37 °C the reaction was stopped by adding 1.9 ml of 0.1 M glycine buffer pH 10.5 and the fluorescence measured using Shimadzu RF 1501 spectrofluorimeter. The concentration of liberated 4MU-NANA product was measured by comparison with a standard calibration curve. One unit of enzyme activity (U) was defined as the conversion of 1 µmol of substrate per min. Similar activity units of each enzyme were used in the assay with LDL: 1 mU of enzyme per 10 µg of LDL.

3.3.4 Lectin blotting

LDL samples were subjected to NuPAGE using 3–8% Novex Bis-Tris gels (Invitrogen) and transferred to a nitrocellulose membrane. The intensity of ApoB protein band stained with Ponceau Red was quantified as a loading control. Blots were blocked in 50 mM Tris-HCl (pH 7.4)

containing 150 mM NaCl, 3% BSA, and 0.05% Tween 20 at room temperature for 1 h and then incubated overnight with biotinylated *Maackia amurensis* lectin (MAL) II, *Sambucus nigra* agglutinin (SNA) or peanut (*Arachis hypogaea*) agglutinin (PNA) (all Vector Laboratories) in the same buffer containing 1% BSA at 4°C. After the washing with TBS-Tween (0.05%), blots were incubated with HRP-conjugated streptavidin for 1 h at room temperature. Following washing, blots were developed using ECL chemiluminescence reagent (Thermo Fisher Scientific).

3.3.5 Analysis of LDL uptake in cultures of human monocyte-derived macrophages

Peripheral blood mononuclear cells were isolated by leukophoresis of blood from human immunodeficiency virus, type 1, and hepatitis B and C seronegative donors followed by centrifugation over Ficoll-Paque Plus (Amersham Biosciences) gradient. Monocytes were isolated from the mononuclear cells using an EasySep™ Human Monocyte Isolation Kit (Stemcell). Cells were plated at a density of 2×10^5 monocytes per cm^2 on glass coverslips in 24-well plates in RPMI 1640 medium containing 10% FBS gold, 1% antibiotic-antimicotic and 20 ng/ml of human recombinant macrophage colony-stimulating factor (M-CSF, eBioscience). After 7 days in culture, differentiated macrophages (larger and more granular than monocytes as seen by light microscopy) were confirmed to have characteristic macrophage cell surface phenotypic markers (CD14, CD206) by flow cytometry.

Human monocyte-derived macrophages were cultured overnight with RPMI medium 1640, containing 5% LPDS and 20 ng/ml M-CSF, supplemented with 30 μg/ml of labeled LDL and incubated at 37°C for 3 h. To study the competition between the uptake of desialylated and oxidized LDL, macrophages were incubated for 3 hours with 30 μg/ml of Dil-labeled oxidized or

desialylated LDL in the absence or in the presence of 5, 10 or 20-fold excess of non-labeled oxidized or desialylated LDL. To study the uptake of LDL by HepG2, the cells grown on glass coverslips coated with poly-l-lysine in 24-well plates were incubated for 20 min in DMEM containing 5% LPDS and 30 µg/ml of native or modified labeled LDL. After the incubation, all cells were washed three times with ice-cold PBS and incubated on ice with 2 mg/ml of heparin (Sigma) in PBS for 2 h. Cells were further rinsed with PBS and fixed for 20 min with 4% paraformaldehyde/4% sucrose solution on ice. The cover slips were mounted on the slides with ProLong® Gold antifade reagent (Thermofisher) and analyzed by the fluorescence microscopy using a Leica DM 5500 Q upright confocal microscope (40x dry objective). Fluorescence intensity of the cells was analyzed for three different randomly selected areas of each cover sleep (~30 cells/area) and quantified using Image J software following previously described method (McCloy et al., 2014).

3.3.6 Analysis of LDL incorporation in the wall of aortic root

Sixteen-week-old *C57BL/6NCrl* male mice kept on a normal diet were injected through the tail vein with 200 µg of native or desialylated Alexa- or Dil-labeled LDL in 100 µl of saline (n=6 animals for each group). Eight hours after injection, mice were euthanized and perfused with 4% paraformaldehyde solution. Accumulation of Alexa or Dil was quantified on cross-sections of the aorta starting at the level of the aortic sinus. For that, isolated hearts were embedded with optimum cutting temperature (OCT) compound (Tissue-Tek). Forty sections with 10-µm thickness were prepared from the top of the left ventricle, where the aortic valves were first visible, up to the position in the aorta where the valve cusps were just disappearing from the field.

Fluorescence was analyzed using a Leica DM 5500 Q upright confocal microscope (40x dry objective). The captured images were quantified using Image J software.

3.3.7 Analysis of atherosclerotic lesions in ApoE$^{-/-}$ mice deficient in neuraminidases 1,3 or 4

To generate *ApoE*$^{-/-}$ mice with deficiencies of neuraminidases 1, 3 and 4, *ApoE*$^{-/-}$ (JaxLab) mice were crossed with previously described *Neu3* KO (*Neu3*$^{-/-}$) (Yamaguchi et al., 2012) or *Neu4* KO mice (*Neu4*$^{-/-}$) (Seyrantepe, Canuel, et al., 2008), or with CathA hypomorph mice with secondary 90% reduction of the Neu1 activity in tissues (*CathA*$^{S190A\text{-}Neo}$) (Seyrantepe, Hinek, et al., 2008). All mice had the same *C57BL/6NCrl* genetic background. Mice were housed in an enriched environment with continuous access to food and water, under constant temperature and humidity, on a 12 h light/dark cycle. Approval for the animal experimentation was granted by the Animal Care and Use Committee of the Ste-Justine Hospital Research Center. Mice were kept on normal chow diet. Between 8 and 20 female mice were analyzed for each genotype.

At the age of 16 weeks mice were sacrificed and areas of atherosclerotic lesions in the aortic root analyzed as described previously (Gayral et al., 2014). Briefly, isolated hearts were washed and incubated overnight in PBS at 4°C, frozen in a cryostat mount with OCT compound (Tissue-Tek) and stored at −80°C. The surface lesion area at the aortic root was measured by computer-assisted image quantification after staining with Oil Red O.

LDL, cholesterol, triglycerides, and HDL levels in the mouse plasma were measured by CHU St-Justine diagnostic biochemistry laboratory using the glycerol phosphate oxidase and

cholesterol esterase assays as described by Allain et al. (Allain, Poon, Chan, Richmond, & Fu, 1974) and Roeschlau et al. (Roeschlau, Bernt, & Gruber, 1974) respectively.

3.3.8 Statistical analysis

Statistical analyses were performed using an unpaired t-test, one-way ANOVA or two-way ANOVA and Prism Graphpad software. The data were first tested for normal distribution using the same software. P-value of 0.05 or less was considered significant. Bonferroni post-hoc test was used to compare specific means, if significance was determined.

3.4 Results

3.4.1 ApoB in human LDL can be desialylated by mammalian neuraminidases 3 and 4.

We first tested if human neuraminidases can remove sialic acid residues from ApoB in LDL in vitro. LDL fraction from healthy human subjects was isolated by preparative ultracentrifugation in the potassium bromide density gradient (from 1.019 to 1.063 g/mL) (Steinbrecher, Witztum, Parthasarathy, & Steinberg, 1987). Purity of the isolated LDL fraction was confirmed by PAGE analysis, which detected a single protein band with a molecular weight of 500 kDa corresponding to that of the major LDL glycoprotein, ApoB. Purified LDL were incubated for 12 h with recombinant human neuraminidases 2, 3 and 4 at the pH corresponding to their pH optimum and the sialylation of ApoB was analyzed by blotting with lectins derived from *Sambucus nigra* (SNA), and *Maackia amurensis* (MAL-II), that have affinity to Sia in α-2,6 and α-2,3 linkages, respectively (Knibbs, Goldstein, Ratcliffe, & Shibuya, 1991; Shibuya et al., 1987) (Figure 1a,b). Our data showed that incubation of LDL with Neu3 resulted in removal of both α-2,3 and α-2,6 attached Sia from the glycan chains of ApoB, whereas Neu4 removed mostly α-2,3 attached Sia chains (Figure 1a). The recombinant human Neu2 was not active against α-2,3 or α-2,6 attached Sia in ApoB glycans (Figure 1b).

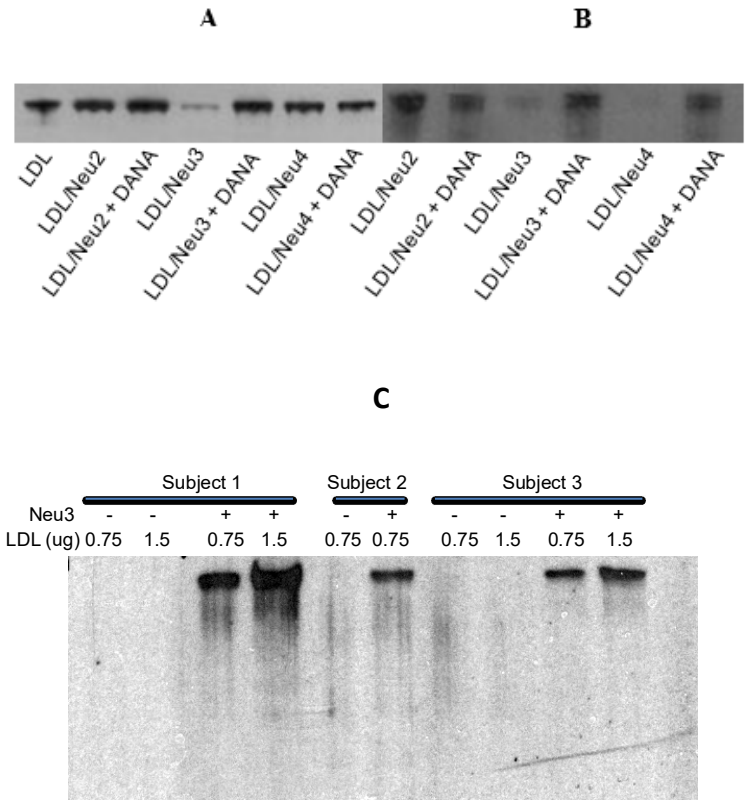


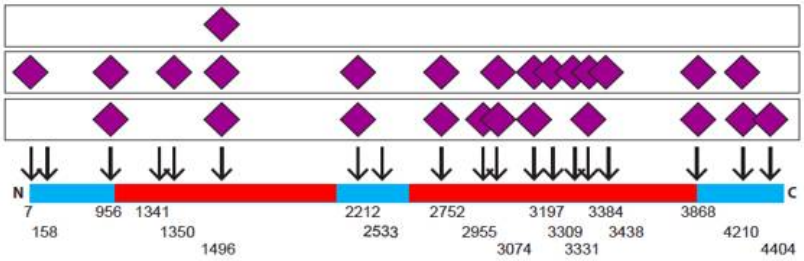
Figure 1. Neuraminidases 3 and 4 remove sialic acids from the glycan chains of ApoB molecule.
Low-density lipoproteins were incubated with human recombinant neuraminidases 2,3 or 4 either with or without the specific neuraminidase inhibitor DANA (2,3-Dehydro-2-deoxy-N-acetylneuraminic acid). The intensity of ApoB protein band stained with Ponceau Red was quantified as a loading control. **A.** Sialylation of ApoB is analyzed by blotting with lectin from Sambucus nigra (SNA) specific to α-2,6 linked Sia. **B.** Sialylation of ApoB is analyzed by blotting with Maackia amurensis lectin (MAL-II) specific for α-2,3 linked Sia. **C.** Treatment with Neu3 results in the recognition of ApoB by PNA specific to carbohydrate sequence Gal-β(1-3)-GalNAc confirming removal of the terminal Sia residues form the glycan chains.

Treatment of LDL with Neu3 also resulted in ApoB staining with peanut agglutinin (PNA) specific to carbohydrate sequence Gal-β(1-3)-GalNAc confirming again the removal of terminal Sia residues from the glycan chains (Figure 1c). Importantly, desialylation of ApoB did not occur in the presence of the specific inhibitor of neuraminidases, N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA, 1 mM concentration in the reaction mixture) or when active Neu3 has been replaced with the mutant enzyme lacking an active site Tyr residue essential for its

activity (Neu3 Y370F) confirming that Sia residues on ApoB are specifically removed by the enzymatic action of neuraminidases.

Structure of the glycan chains of ApoB in native LDL or those treated with Neu3 was further analyzed by tandem mass spectroscopy. For this, the LDL proteins were resolved by PAGE and gel pieces containing ApoB bands were treated with endoglycosidase, PNGase F. The released ApoB N-glycans were extracted from the gel pieces and their structure was determined using Waters Q-TOF Premier nanoACQUITY UPLC-MS/MS instrument with an ESI source. The identification of peptides containing N-glycosylation was performed after in-gel trypsin digestion of ApoB followed by extraction of peptides and their analysis by LC-MS/MS. Analysis of ApoB glycan chains has shown that Neu3 removes Sia from the complex glycan chains linked to Asn1523, Asn2976, Asn3095, Asn3353 and Asn4488/4489 residues, reducing the total sialylation of glycan chains from 96% to 36% (Table 1).

Table 1. MS/MS. Desialylation of LDL ApoB glycan chains by recombinant human Neu3



Sample	Peptide mass (Da)	Peptide sequence	Glycosylation site	Glycan composition
Control ApoB100 2014-08-05 2014-08-07	279.122	(R)FN(S)	N1523	NeuAc1Hex5HexNAc4
	279.122	(L)NF(S	N2982	NeuAc1Hex5HexNAc4
	279.122	(Q)NF(S)	N3102	NeuAc1Hex5HexNAc4
	279.122	(L)FN(Q)	N3358	NeuAc1Hex5HexNAc4
	366.154	(R)FNS(S)	N1523	NeuAc1Hex5HexNAc4
	366.154	(L)NFS(K)	N2982	NeuAc1Hex5HexNAc4
	366.154	(Q)NFS(A)	N3101	NeuAc1Hex5HexNAc4
	366.154	(D)FNS(S)	N3465	NeuAc1Hex5HexNAc4
	407.180	(N)QNF(S)	N3102	NeuAc1Hex5HexNAc4
	407.180	(L)FNQ(S)	N3358	NeuAc1Hex5HexNAc4
	453.186	(G)AYSN(A)	N983	NeuAc1Hex5HexNAc4
	453.186	(A)YSNA(S)	N983	NeuAc1Hex5HexNAc4
	453.186	(R)FNSS(Y)	N1523	NeuAc1Hex5HexNAc4
	453.186	(D)FNSS(M)	N3465	NeuAc1Hex5HexNAc4
	467.202	(S)NFTS(Q)	N4431	NeuAc1Hex5HexNAc4
	494.213	(N)QNFS(A)	N3101	NeuAc1Hex5HexNAc4
	494.213	(L)FNQS(D)	N3358	NeuAc1Hex5HexNAc4
	494.212	(Q)NFSAG(N)	N3101	NeuAc1Hex5HexNAc4
	568.213	(Y)DFNSS(M)	N3465	NeuAc1Hex5HexNAc4
	607.297	(E)LFNQS(D)	N3358	Hex9HexNAc2
	612.239	(K)SYNET(K)	N3224	NeuAc2Hex5HexNAc4
	612.239	(K)SYNET(K)	N3224	NeuAc1Hex5HexNAc4
	621.276	(E)NIDFN(K)	N2239	NeuAc1Hex5HexNAc4
	646.292	(N)ADIGNGT(T)	N3465	NeuAc1Hex5HexNAc4
	836.414	(S)NLRFNSS(Y)	N1523	NeuAc1Hex5HexNAc4
	836.414	(E)SNLRFNS(S)	N1523	NeuAc1Hex5HexNAc4
	836.414	(S)NLRFNSS(Y)	N1523	NeuAc2Hex5HexNAc4
	836.414	(E)SNLRFNS(S)	N1523	NeuAc2Hex5HexNAc4
Neu3-treated ApoB100 2014-08-05	279.122	(R)FN(S)	N1523	NeuAc1Hex5HexNAc4
	279.122	(L)NF(S	N2982	NeuAc1Hex5HexNAc4
	279.122	(Q)NF(S)	N3102	NeuAc1Hex5HexNAc4
	279.122	(L)FN(Q)	N3358	NeuAc1Hex5HexNAc4
	407.180	(N)QNF(S)	N3102	Hex5HexNAc4
	407.180	(L)FNQ(S)	N3358	Hex5HexNAc4
	437.158	(Q)NFSA(G)	N3101	Hex5HexNAc4
	453.186	(G)AYSN(A)	N983	NeuAc1Hex5HexNAc4
	453.186	(A)YSNA(S)	N983	NeuAc1Hex5HexNAc4
	453.186	(R)FNSS(Y)	N1523	NeuAc1Hex5HexNAc4
	453.186	(D)FNSS(M)	N3465	NeuAc1Hex5HexNAc4
	460.228	(D)IGNGT(T)	N2779	NeuAc1Hex5HexNAc4
	467.202	(S)NFTS(Q)	N4431	Hex5HexNAc4
	494.213	(N)QNFS(A)	N3101	Hex5HexNAc4
	494.213	(L)FNQS(D)	N3358	Hex5HexNAc4
	494.212	(Q)NFSAG(N)	N3101	Hex5HexNAc4
	568.213	(Y)DFNSS(M)	N3465	Hex5HexNAc4
	575.255	(F)NQSDI(V)	N3358	Hex5HexNAc4
	611.255	(G)AYSNAS(S)	N983	Hex5HexNAc2
	612.239	(K)SYNET(K)	N3224	Hex5HexNAc4
	621.276	(E)NIDFN(K)	N2239	Hex5HexNAc4
	663.282	(G)NGTTSAN(E)	N2779	NeuAc1Hex5HexNAc3
	665.313	(Y)KYNQN(F)	N3101	Hex5HexNAc4
	665.313	(Y)KYNQN(F)	N3101	Hex5HexNAc3
	699.253	(Y)DFNSSM(L)	N3465	Hex4HexNAc3
	699.253	(Y)DFNSSM(L)	N3465	Hex5HexNAc4
	836.414	(S)NLRFNSS(Y)	N1523	Hex5HexNAc4
	836.414	(E)SNLRFNS(S)	N1523	Hex5HexNAc4

Summary of changes in sialic content of human ApoB

Sample	*Number of glycopeptides*	*Total #Sia residues*	*% containing Sia*	*Avg # Sia/glycan*
Control	28	30	96%	1.1
Neu3-treated	28	10	36%	0.4

3.4.2 Desialylation of LDL increases their uptake by cultured human monocyte-derived macrophages.

To test whether removal of sialic acids from glycan chains of ApoB affects the uptake of LDL by macrophages we compared the uptake of fluorescently labeled native LDL, LDL desialylated by Neu3 treatment (desLDL) or oxidized LDL (oxLDL) by cultured human blood monocyte-derived macrophages. Two types of labels were used, Alexa Fluor, that covalently binds to the ApoB molecule, and DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), that incorporates into the lipid core of LDL due to its hydrophobicity. Using two types of labeling allowed us to analyze simultaneously the uptake of both the LDL particles and cholesterol. Human blood monocytes were purified from the blood of healthy volunteers and differentiated into macrophages by culturing for 7 days in the presence of M-CSF. To test the uptake, the cells cultured on glass coverslips were starved overnight in the medium containing human lipoprotein-deficient serum instead of FBS. Then the cell medium was supplemented with either modified or native LDL and cells were incubated for 3 h at 37^{o}C, washed, fixed and studied by fluorescent microscopy. Our data (Figure 2) show that incubation of human macrophages with desLDL, resulted in significantly higher accumulation of both Alexa and DiI labels as compared with the cells incubated in the presence of similarly labeled native LDL. Desialylated LDL were engulfed at a rate similar or even higher than that for oxidized LDL (Figure 2). Together the data

suggested that removal of Sia from the ApoB by neuraminidases dramatically increases their uptake by cultured macrophages.

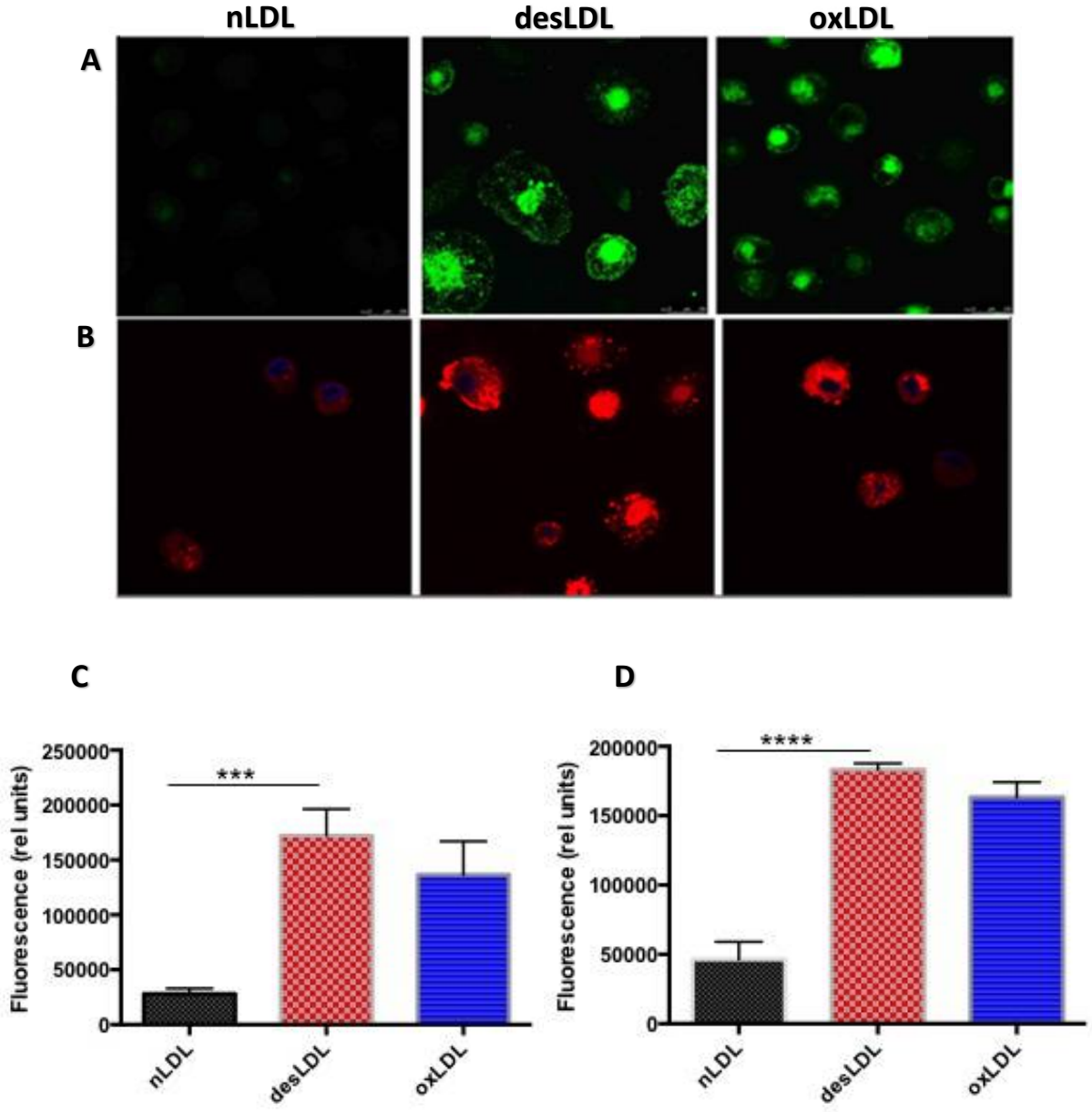


Figure 2. Desialylation of LDL increases their uptake by human monocyte-derived macrophages.
Human monocyte-derived macrophages were incubated with 30 µg/ml of labeled LDL for 3 h at 37°C. After incubation cells were washed, fixed and analyzed by confocal microscopy. Relative fluorescence intensities of the cells were measured by Image J software. Enzymatically desialylated LDL in contrast to native LDL are engulfed by cultured monocyte-derived macrophages at a rate comparable or even higher than that for oxidized LDL. **A**. Accumulation of Alexa-labeled LDL. **B**. Accumulation of Dil-labeled LDL**.** Bar graphs show average fluorescence intensities of cells treated with Alexa-labeled (**C)** and Dil-labeled LDL (**D**). Data show mean values ± SD of three independent experiments. (*** $p < 0.001$ as compared with native LDL by t-test).

3.4.3 Desialylation of LDL does not change the rate of their uptake by HepG2 cells.

To analyze if desialylation of ApoB affects affinity of LDL towards hepatocyte LDL receptor we tested the uptake of native LDL and desLDL by cultured human liver carcinoma cells, HepG2. Similarly, to primary hepatocytes, HepG2 cells express high levels of LDLR and are routinely used to study the LDL uptake by LDLR-mediated pathway. Cultured HepG2 were incubated for 20 min in the presence of 30 μg/ml of native or desialylated LDL labeled with Alexa or DiI and then the uptake of the dye into the cells was quantified as described above for macrophages. Our data show that native and desialylated LDL were taken up by cultured HepG2 cells at a similar rate (Figure 3) indicating that desialylation of LDL did not change their affinity to LDL receptors.

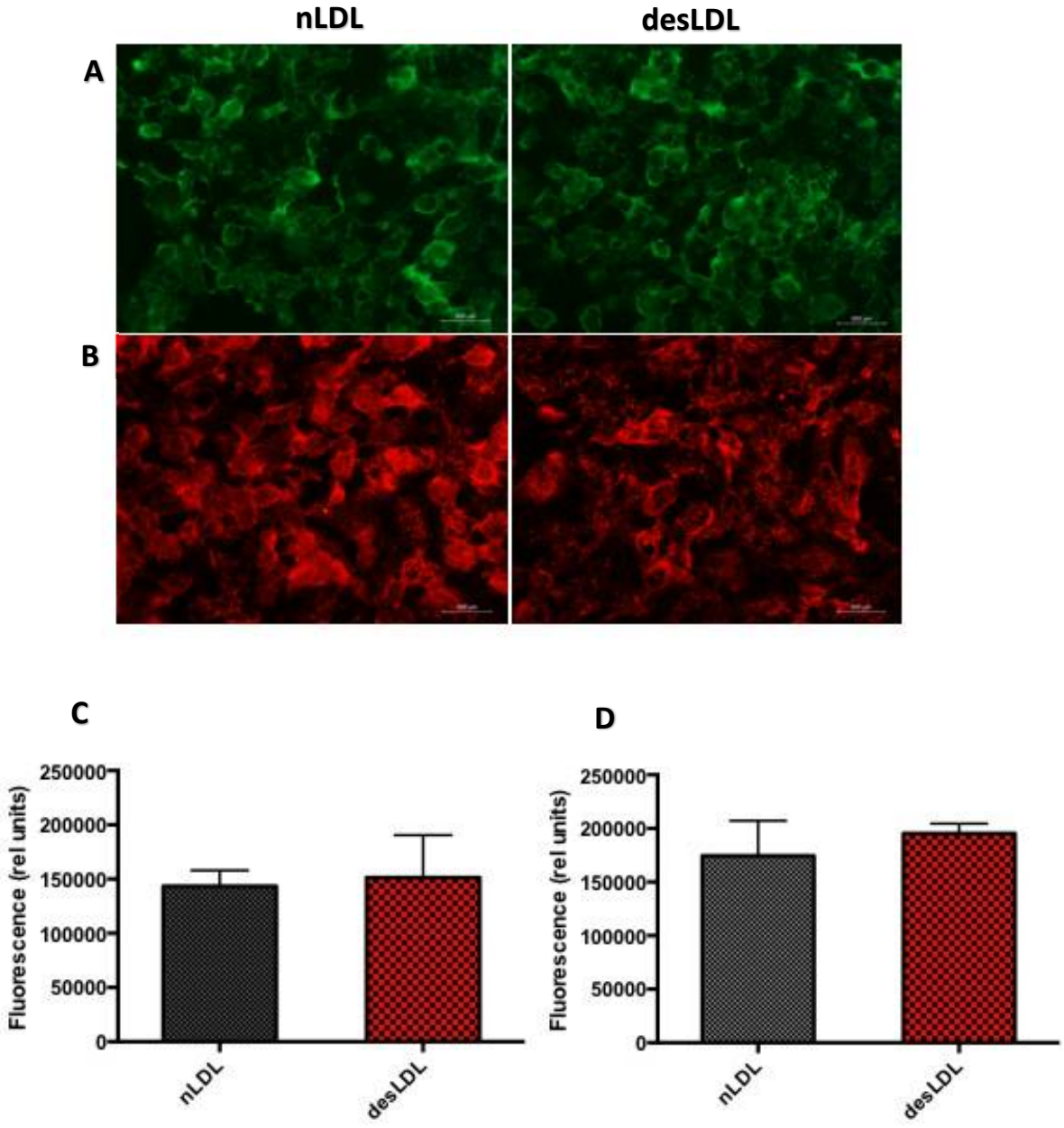
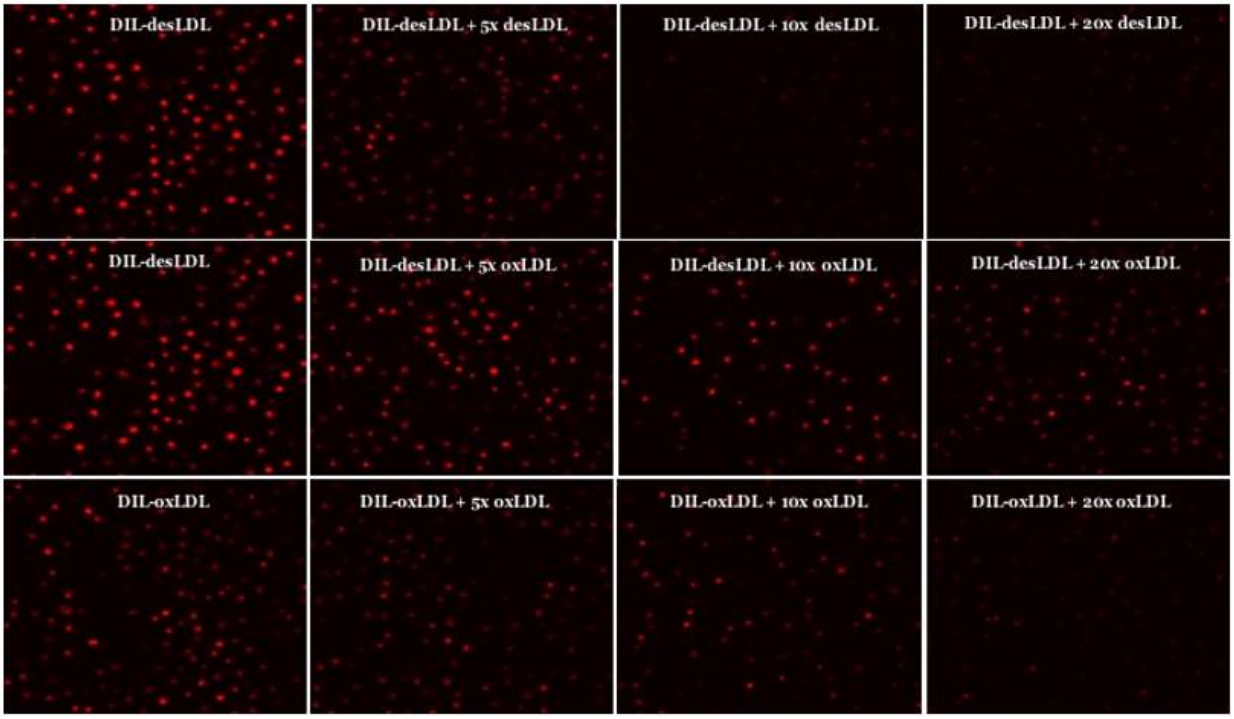


Figure 3. Desialylation of LDL does not affect their uptake by cultured hepatocytes.

HepG2 cells were incubated with 30 µg/ml of labeled LDL for 20 min at 37°C, and then after washing and fixation analyzed by fluorescent microscopy. Relative fluorescence intensities of the cells were measured by ImageJ software**. A**. Accumulation of Alexa-labeled LDL. **B.** Accumulation of DiI-labeled LDL. Bar graphs show average fluorescence intensities of cells treated with Alexa-labeled (**C)** and DiI-labeled LDL (**D**). Data show mean values ± SD of three independent experiments.

3.4.4 The uptake of desialylated LDL can be inhibited by oxidized LDL

In order to study if endocytosis of desialylated and oxidized LDL occurs through the same or different surface receptors we incubated human monocyte-derived macrophages with 30 μg/mL of DiI-labeled desLDL and a 5, 10 or 20 times excess of unlabeled desialylated or oxidized LDL. As before, the cells were analyzed by confocal microscopy and the fluorescence intensity quantified with Image J software. Our data (Figure 4) show that the uptake of labeled fluorescent desLDL was completely blocked by the excess of unlabeled desLDL, but only partially inhibited by the excess of unlabeled oxLDL. This indicates that endocytosis of desLDL occurs through several pathways both common for desialylated and oxidized LDL and those specific for desialylated particles.



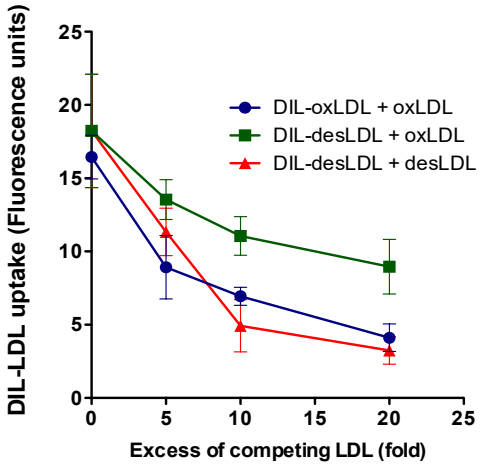


Figure 4. Oxidized LDL only partially block the uptake of desialylated LDL by macrophages

Cultured macrophages where incubated for 3 h at 37°C with Dil-labeled desialylated or oxidized LDL with or without 5, 10 or 20-fold excess of non-labeled oxidized or desialylated LDL. Cells were washed, fixed and analyzed by fluorescence microscopy. Relative fluorescence intensities of the cells were measured by Image J software. Data show mean values ± SD of three independent experiments. Inhibition of desLDL uptake by oxLDL is significantly different from that by desLDL ($P < 0.01$).

3.4.5 Desialylation of LDL increases its incorporation into the mouse aortic root wall after systemic injection.

We further studied if in addition to increasing the LDL uptake by macrophages in vitro desialylation also increases their incorporation into the arterial wall in live mice. For that, sixteen weeks old *C57BL/6NCrl* male mice were injected via the tail vein with 200 µg of native or desialylated LDL fluorescently labeled with Alexa or DiI and dissolved in 200 µl of saline. Control mice were injected with the same volume of saline only. Eight hours after injection mice were sacrificed, their hearts collected and aortic roots analyzed for the presence of LDL particles in the aortic wall. The OCT-embalmed blocks with aortic roots were sectioned on cryostat and 10-µm thick slices analyzed by confocal microscopy (Figure 5). The quantification of the fluorescence intensity with Image J software demonstrated that the incorporation of Alexa or DiI dye into the aortic wall was significantly higher (4 and 2 times, respectively, $P < 0.05$) when mice were injected with desialylated LDL as compared with those injected with native LDL.

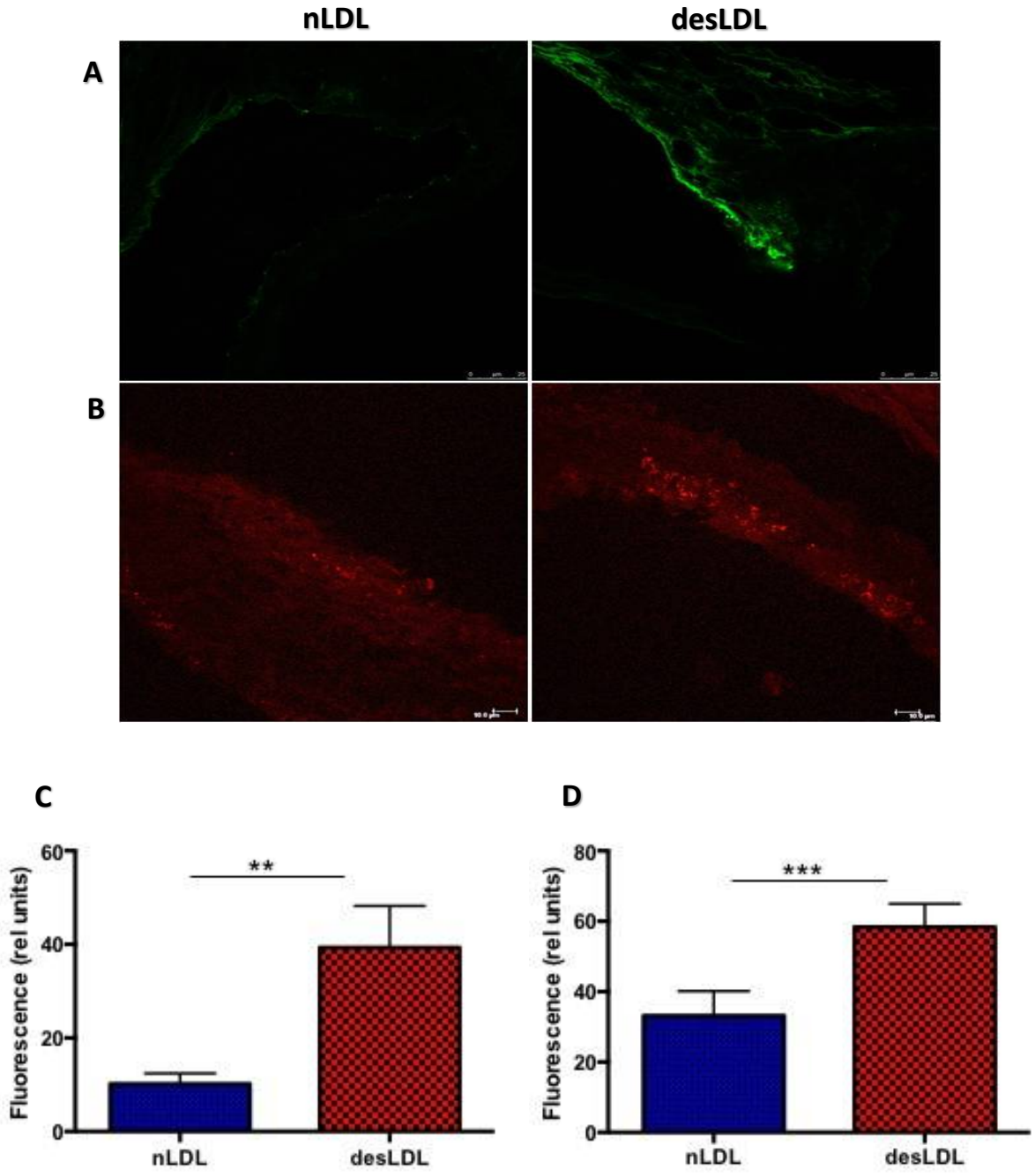


Figure 5. Desialylation increases incorporation of LDL into the mouse aortic wall.

Accumulation of native and desLDL in the aortic root wall of mice was studied 8 h after injection of 200 μg of labeled LDL into their tail vein. Sectioned aortic root was analyzed by fluorescent confocal microscopy. Relative fluorescence intensities were measured by Image J software. **A**. Accumulation of Alexa-labeled nLDL and desLDL. **B**. Accumulation of DiI-labeled nLDL and desLDL. **C** and **D** show the results of quantification of Alexa and DiI dye in the aorta wall. Data show mean values ± SD of three independent experiments. (**, *** significantly different from nLDL uptake; $P < 0.01$ and <0.001 respectively).

3.4.6 Early stage of atherosclerosis is delayed in gene-targeted neuraminidase 1 and neuraminidase 3 deficient mice.

To evaluate whether neuraminidases play a role in atheroma progression in vivo we performed genetic inactivation of individual neuraminidases in Apolipoprotein E-knockout mice (*ApoE*$^{-/-}$), the commonly used spontaneous murine model of atherosclerosis. As compared with WT *C57BL/6NCrl* mice *ApoE*$^{-/-}$ mice have significantly increased level of a total cholesterol and LDL cholesterol in blood (Jawien et al., 2004) even when they are fed a regular diet. Approximately at the age of 15 weeks they develop intermediate aortic lesions containing both foam and smooth muscle cells (Meir & Leitersdorf, 2004). *ApoE/Neu3* and *ApoE/Neu4* double-knockout mice were generated by crossing homozygous *ApoE*$^{-/-}$ mice with homozygous *Neu3* and *Neu4* KO mice, respectively (Seyrantepe, Canuel, et al., 2008; Yamaguchi et al., 2012). To assess the role of Neu1 in atherosclerosis, we crossed *ApoE*$^{-/-}$ mice with previously described cathepsin A-hypomorph mice (*CathA*$^{S190A\text{-}Neo}$). These mice have a ~90% reduction in Neu1 activity in tissues (Seyrantepe, Hinek, et al., 2008) but do not develop a rapidly progressing neurologic disease occurring in a *Neu1* KO due to the lysosomal storage of sialoglycoconjugates in neurons (de Geest et al., 2002), which makes the *Neu1* KO strain unsuitable for physiological studies. All mice had the same *C57BL/6NCrl* background, were fertile and had normal development with the increase of body weight with age similar to that of normal mice. In order to analyze if neuraminidases are involved in the initial stage of atherosclerosis we kept mice on a normal diet and sacrificed them at 16 weeks when the intermediate lesions already become apparent in the aortic root. After sacrifice mouse hearts were collected, frozen in OCT-embalmed blocks and the aortic root regions were sectioned into 10-µm thick slices. Analysis of lipid deposition in the aortic root

sections of control *ApoE*$^{-/-}$ female mice showed fatty streak lesions with a significantly higher average area than in *ApoE*$^{-/-}$*CathA*$^{S190A\text{-}Neo}$ female mice ($P < 0.05$) (Figure 6). Average lesion size of *ApoE*$^{-/-}$ females was 177000 ± 9900 µm^2 vs. average lesion size of 125000 ± 6000 µm^2 for *ApoE*$^{-/-}$ *CathA*$^{S190A\text{-}Neo}$ mice. The mean size of lesions in *ApoE*$^{-/-}$*Neu3*$^{-/-}$ mice was also significantly smaller than that in *ApoE*$^{-/-}$ mice although variability between the individual lesion size values in *ApoE*$^{-/-}$ *Neu3*$^{-/-}$ animals was higher than that in *ApoE*$^{-/-}$*CathA*$^{S190A\text{-}Neo}$ mice. This result clearly demonstrates that deficiency of Neu1 or Neu3 is associated with dramatic reduction of the lesion size in *ApoE*$^{-/-}$ mice. At the same time, the size of atherosclerotic lesions in *ApoE*$^{-/-}$*Neu4*$^{-/-}$ was similar to that in *ApoE*$^{-/-}$ animals. No significant difference was found in the progression of atherosclerosis between *ApoE*$^{-/-}$ and double mutant male mice.

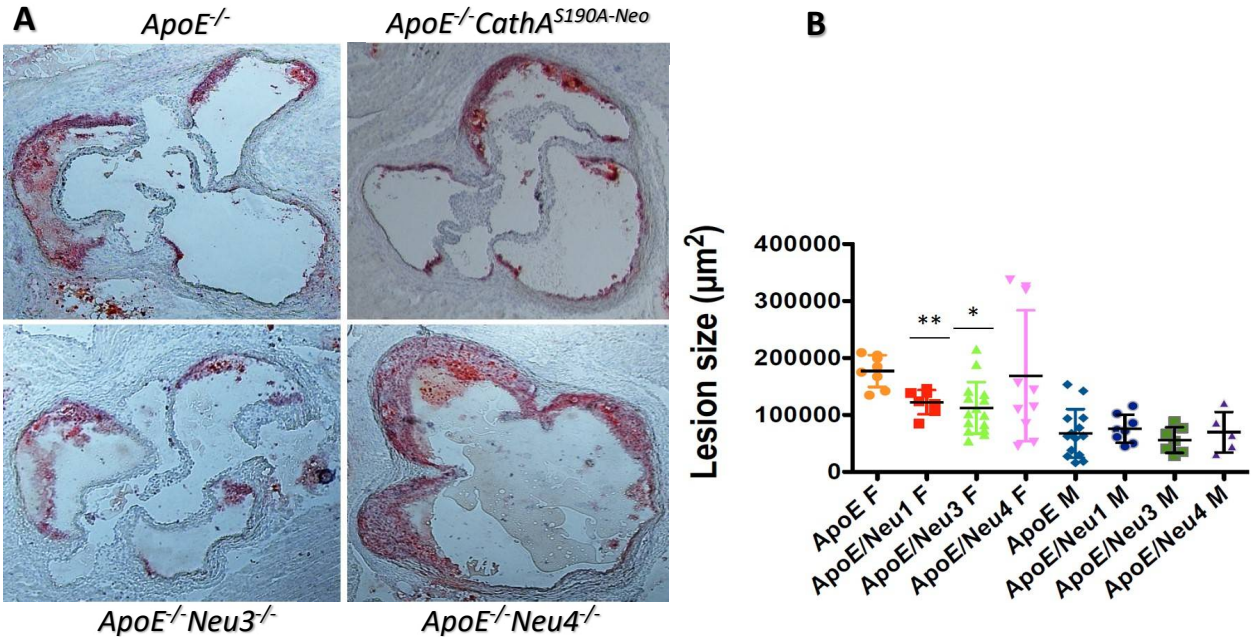


Figure 6. Reduced size of fatty streaks in the aortic root of female *ApoE*$^{-/-}$ mice deficient in Neu1.
ApoE$^{-/-}$ mice (n=8) and *ApoE*$^{-/-}$ mice deficient in Neu1 (n=7), Neu3 (n=15) or Neu4 (n=10), were fed with normal diet and sacrificed at the age of 16 weeks. Atherosclerosis was analyzed by staining fatty streaks in the thin sections of mouse aortic roots with Red Oil O. **A.** Representative images of aortic root sections from female mice stained with Red Oil O. **B.** Bar graph showing atherosclerotic lesion size in the aortic roots (µm^2) measured by Image J software.

The analysis of female mouse plasma did not reveal any significant differences in the levels of total cholesterol, LDL cholesterol, HDL-cholesterol or triglycerides between *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*CathA*$^{S190A\text{-}Neo}$ mice (Figure 7) suggesting that the decrease in the size of the atherosclerotic lesions in female *ApoE*$^{-/-}$*CathA*$^{S190A\text{-}Neo}$ mice was not caused by changes in plasma cholesterol levels. HDL cholesterol levels were increased in the *ApoE*$^{-/-}$*Neu4*$^{-/-}$ mice whereas both total cholesterol level and LDL-cholesterol level were slightly reduced in Neu3-deficient mice. Physiological importance of these changes and their relation to reduced atherosclerotic lesions in *ApoE*$^{-/-}$*Neu3*$^{-/-}$ mice still remains to be studied.

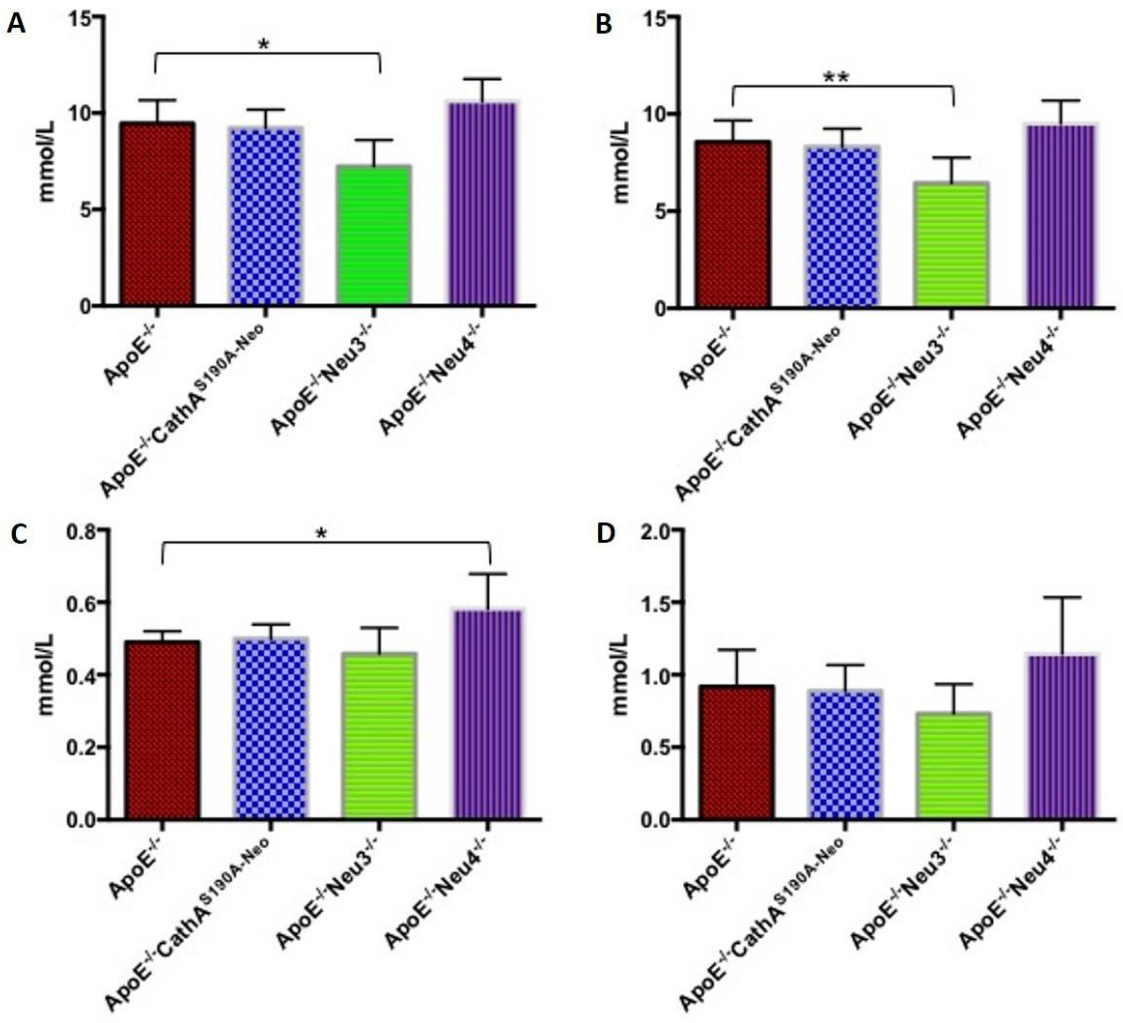


Figure 7. Lipid analysis in female mouse plasma.

Total cholesterol (**A**), LDL cholesterol (**B**), HDL cholesterol (**C**) and triglyceride (**D**) levels were measured in mouse plasma samples. Data represent means ± SEM (*, ** significantly different, $P < 0.05$, $P < 0.01$) as compared with *ApoE*$^{-/-}$ mice.

Finally, the sialylation of ApoB in the plasma of Neu1-deficient mice that showed slower rate of atherosclerosis was analyzed by lectin blot. Proteins from LDL fraction isolated from pooled blood obtained by cardiac puncture from ten 16 week-old *ApoE*$^{-/-}$ or *ApoE*$^{-/-}$*CathA*$^{S190A\text{-}Neo}$ mice were resolved by SDS PAGE, transferred to a nitrocellulose membrane and blotted with biotinylated SNL as described above for the human LDL fraction (Figure 8). Quantitation of the

intensity of lectin-stained ApoB bands showed that ApoB sialylation in the blood of *ApoE*$^{-/-}$ *CathA*$^{S190A\text{-}Neo}$ mice was significantly increased as compared with that in *ApoE*$^{-/-}$ group.

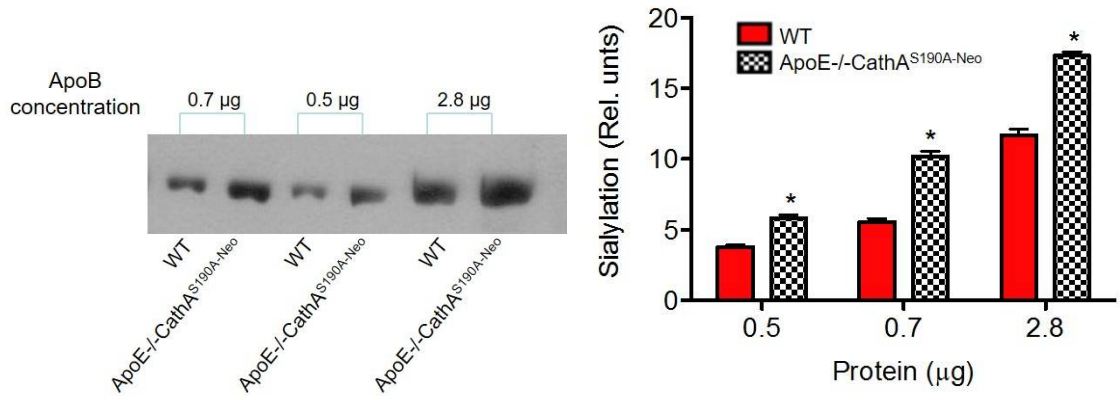


Figure 8. Increased sialylation of LDL ApoB in the blood of Neu1-deficient mice.
Blood was collected by cardiac puncture into EDTA-coated tubes from ten 4-5 months-old *Neu1* KI (*CathA*$^{S190A\text{-}Neo}$) mice and the same number of WT control mice with matching age and sex. For each group LDL was isolated from 4 mL of pooled plasma by sequential density gradient ultracentrifugation (1.019 to 1063 g/mL) as has been described previously. Sialylation of the major LDL protein, ApoB-100 (~500 kDa) was analyzed by blotting using biotinylated Sambucus nigra lectin (Vector Labs; dilution 1: 20,000).
The upper panel shows image of a representative blot performed with 0.7, 0.5 and 2.8 µg of LDL protein. The graph below shows results of quantification (mean values ±SD) performed on 3 individual blots by Image J software. *Significantly different from WT ($P<0.05$) according to one-way ANOVA test.

3.5 Discussion

The major role in atherogenesis is played by biochemically modified LDL, which have a longer circulation time due to a reduced affinity to LDLR (Orekhov et al., 1992) and can provoke a cascade of responses that lead to atherogenesis in a previously unaffected healthy artery. Desialylation of LDL was proposed to be one of such changes because it would increase the ApoB affinity to scavenger asialoglycoprotein receptors and cellular surface proteoglycans in

macrophages (Orekhov et al., 1992). Desialylation of LDL could also lead to their aggregation, which was suggested to be a key factor for intracellular lipid accumulation (Musliner et al., 1987). In the current study we provide direct evidence that neuraminidases 1 and 3 play pathological roles in atherosclerosis by catalyzing desialylation of LDL and increasing their atherogenisity in vivo.

We showed that human recombinant Neu3 reduces total sialylation of the glycan chains of ApoB from 96% to 36% and that this results in LDL accumulation by macrophages at a rate comparable or even higher than that for oxidized LDL. Previously it has been proposed that the uptake of desialylated LDL by macrophages occurs via scavenger receptors, the same as for oxLDL (Kakino, Fujita, Nakano, Horiuchi, & Sawamura, 2016; Orekhov et al., 1992). However, we showed that accumulation of desLDL by macrophages was only partially inhibited by the excess of oxLDL, suggesting the existence of an additional pathway(s) for their endocytosis. DesLDL have exposed Gal residues (Taniguchi et al., 1989), which should make them a high affinity ligand for the cellular lectin receptors specific for β-galactose (Gal) residues (Lee et al., 1983). Moreover, lipopolysaccharide-stimulated macrophages express increased amounts of Gal/GalNAc-specific lectin and show increased uptake of desLDL (Grewal et al., 1996). We hypothesize therefore that the increased uptake of desLDL by macrophages in addition to the scavenger receptor-mediated pathway most likely occurs via galactose-specific lectin receptors such as asialoglycoprotein receptor, that are elevated under inflammatory conditions (Bartlett et al., 2000).

According to our data desialylation resulted in the augmented uptake of LDL by human cultured macrophages as well as their increased accumulation in the aortic root wall in live mice. Furthermore, our in vivo experiments showed that desialylation of LDL leads to their

accumulation in the arterial wall in live mice. Analysis of lipid deposition in the aortic root of double-mutant mice showed that decreased activity of Neu1 and Neu3 causes a dramatic decrease of the lesion size in *ApoE* KO female mice, but not in the male group. In the *ApoE* KO mice male animals have significantly slower progression of atherosclerosis lesions as compared with females (Meyrelles, Peotta, Pereira, & Vasquez, 2011). We suggest therefore, that the effect of neuraminidase deficiency on the atherosclerotic plaque progression in the male mice has to be analyzed at an older age such as at 5 or 6 months. In contrast to Neu1 and Neu3, genetic deficiency of Neu4 does not change the size of atherosclerotic lesions in both female and male *ApoE* KO mice, suggesting that this enzyme is not involved in the development of atherosclerosis. These correlates well with the data of our in vitro experiments that show that Neu4 causes only partial desialylation of human LDL.

The analysis of mouse plasma showed that Neu3 deficiency causes a small but significant reduction of total and LDL cholesterol. The physiological importance of these changes and their relation to reduced atherosclerotic lesions in *ApoE/Neu3* KO mice still remains to be studied. At the same time decreased activity of Neu1 did not affect the level of total cholesterol, suggesting that the decrease in the size of the atherosclerotic lesions was not caused by changes in plasma cholesterol levels, but rather by increased sialylation of ApoB which we directly demonstrated by lectin blot. Considering that both Neu1 and Neu3 are induced in monocytes during their differentiation into macrophages and further during the activation of macrophages it is tempting to speculate that the increase in circulating desialylated LDL occurs in response to inflammation associated with atherosclerosis resulting in a vicious cycle: LDL desialylation → LDL uptake → recruitment/activation of further macrophages → induction of Neu1/3 → LDL desialylation →

LDL storage. Overall, our data demonstrate that changes in LDL sialylation can cause effects strong enough to influence predisposition to atherosclerosis, suggesting that leucocyte neuraminidases 1 and 3 play an essential role in triggering atherosclerosis. In turn, this allows to hypothesize that neuraminidase 1 and 3 inhibitors can be potential candidates for treating atherosclerosis in human patients.

Chapter 4. General Discussion and Future directions

4.1 Overview

Atherosclerosis is a leading cause of death and loss of productive life years worldwide. Research into the disease has led to many compelling hypotheses about the pathophysiology of atherosclerosis and its complications such as myocardial infarction and stroke. However, although recent research advances have helped to unravel some of the principles of atherosclerosis pathophysiology, gaps remain in translation to the clinic. Observational data support a strong association between plasma LDL levels and augmented individual susceptibility to atherosclerosis and its complications (Bentzon et al., 2014; Keys, 1997; Martin et al., 1986). Lowering LDL levels by inhibitors of hydroxymethyl glutaryl coenzyme A reductase, which are collectively known as statins and compose the current mainstay of therapeutic management of atherosclerosis diminish the likelihood of atherosclerotic events (Grundy, 2002). At the same time, in CAD statins only benefit ∼35% of patients (Arsenault et al., 2012). Moreover >20% of patients will also have a recurrent event within 30 months of an acute coronary syndrome, despite receiving high-dose statin treatment (Morrissey, Diamond, & Kaul, 2009) . Antioxidant supplements such as vitamins E and C alone provided no benefit to CAD patients in secondary prevention (Cherubini et al., 2005), except in the Fukuoka Atherosclerosis trial (FAST), where probucol lowered the incidence of cardiac events (Sawayama et al., 2002). Finally, despite experimental evidence that inflammation has a key role and transduces the effects of many known risk factors for atherosclerosis, many traditional anti-inflammatory therapies (glucocorticoids, non-steroidal anti-inflammatory drugs, certain PPAR agonists or TNF inhibitors) (Charo & Taub, 2011; Grundy, 2002; Kearney et al., 2006; Okuyama et al., 2015) do not improve

cardiovascular outcomes, and some may even aggravate atherosclerotic events, whereas 'cardioprotective' effect of aspirin (50–150 mg daily) is probably caused by its antiplatelet rather than anti-inflammatory action (Patrono, Coller, FitzGerald, Hirsh, & Roth, 2004). Altogether, the above evidences underline necessity of further insight into atherosclerosis leading to new clinical applications.

Our current data define a novel pathway contributing to atherosclerosis, desialylation of LDL by human monocyte neuraminidases 1 and 3 and provide evidence that the inhibitors of these enzymes can be therapeutically explored for reducing development of atherosclerotic lesions.

4.2 Analysis and future directions

Human neuraminidases encoded by four genes (*NEU1 – NEU4*) play important roles in various cellular functions. Each neuraminidase plays a unique role depending on its enzyme properties, but many of them remained underexplored. Understanding of substrate preferences of neuraminidases is providing clues for controlling the level of sialylation of functional molecules. In the first study, we performed the analysis of the substrate specificity of 4 human neuraminidases. We measured the kinetics of hydrolysis of BODIPY-labeled (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid) substrates containing common mammalian sialylated oligosaccharides: 3'SiaLacNAc, 3'SiaLac, SiaLex, SiaLea, SiaLec, 6'SiaLac, and 6'SiaLacNAc. By analyzing Vmax and Km values of the enzymatic reactions we found that neuraminidases discriminate between different sialoside targets.

Neu1 showed the broadest substrate preferences, which corresponds to its wide diversity in the biological functions. Both Neu1 and Neu3 readily cleaved α2,6-linked Sia. Since the major LDL surface glycoprotein, apolipoprotein B 100 (ApoB) on average contains 12-14 α2,6-linked Sia residues (10% of the total carbohydrate content) our experiments suggested that atherogenic LDL can be produced by removal of terminal sialic acid residues (Sia) from ApoB glycan chains by the action of neuraminidases 1 and 3.

It is now recognized that a major role in atherogenesis is played by biochemically modified LDL, which have a longer circulation time due to a reduced affinity to LDLR (Orekhov et al., 1992) and can provoke a cascade of responses that lead to a disease in a previously unaffected healthy artery. In particular, desialylation of LDL causes physical and chemical modifications in the ApoB structure, which increases its affinity to scavenger asialoglycoprotein receptors and cellular surface proteoglycans in macrophages. According to our data desialylation results in the augmented uptake of LDL by human cultured macrophages as well as their increased accumulation in the aortic root wall in live mice. Importantly, in contrast to oxidation, desialylation is a naturally occurring modification of LDL. Since LDL is constantly retroendocytosed via acidic endosomes (Berg, Gjoen, & Bakke, 1995), and might come into contact with neuraminidases intracellularly or on the cell surface, it is possible that oligosaccharide chains on ApoB become progressively desialylated with time (Schonfeld & Krul, 1986). This might explain the large amount of desialylated oligosaccharides found in LDL of CAD patients. Previous publications also showed that high blood levels of free Sia are an indication of atherosclerosis and CAD and that total Sia content in LDL from the blood of CAD patients is reduced as compared to healthy controls (Orekhov et al., 1991; Tertov et al., 1993). Considering

that both Neu1 and Neu3 are induced in monocytes during their differentiation into macrophages and further during activation of macrophages it is tempting to speculate that the increase in circulating desialylated LDL occurs in response to inflammation associated with atherosclerosis resulting in a vicious cycle: LDL desialylation → LDL uptake → recruitment/activation of further macrophages → induction of Neu1/3 → LDL desialylation → LDL storage. In addition, published data show that atherosclerotic lesions are frequently found in low-sialylated areas of aortic endothelium, and removal of Sia from the intima of aorta by neuraminidases increases the adhesion of circulating platelets as well as the uptake of LDL (Tertov et al., 1992) suggesting that neuraminidases on endothelial cells could activate the first steps of the atherogenesis, the appearance of the ICAM-1 adhesion molecules on the surface of endothelial cells, leading to an increase in binding and infiltration of leucocytes. Both Neu1 and Neu3 are expressed in endothelial cells and Neu1 overexpression in human lung endothelial cells reduces their migration (Cross et al., 2012). It has also been demonstrated that removal of Sia from β2-integrin (CD18 and CD11b) and ICAM-1 by polymorphonuclear leukocyte (PMN) neuraminidase (presumably Neu1) exposes activation epitopes on each of these binding partners enhancing their interaction and resulting in tight adhesion between PMNs and endothelial cells (Feng et al., 2011). Therefore, there are multiple processes by which neuraminidases 1 and 3 could affect certain steps in atherogenesis, making them a valid target for treating atherosclerosis.

Since the removal of terminal Sia from the glycan chains on ApoB exposes β-linked galactose residues (Gal) we suggest that desLDL may also bind to galectin 3 (MAC-2), the abundant Gal-binding lectin implicated in the pathophysiology of CAD and highly present on

intimal macrophages and within atherosclerotic lesions in mice and humans. Recent studies showed that *ApoE/Gal3* knockout mice fed with a high-cholesterol diet had similar serum lipid profile as single *ApoE* KO mice but showed significantly reduced formation of atherosclerotic lesions in the thoracic aorta (57% reduction), the aortic arch (50% reduction) and the brachiocephalic arteries. The aortic plaques were smaller, with reduced lipid core and collagen. Moreover, administration of modified citrus pectin, an inhibitor of galectin-3, during the latter stage of the disease also reduced plaque volume in *ApoE* KO mice. We speculate therefore that desialylation of LDL by monocyte neuraminidases and their uptake via macrophage lectin receptors can constitute a novel pathway for the development of atherosclerosis.

Neu1 and Neu3 are localized at the lysosomal membranes (Winter, Swallow, Baraitser, & Purkiss, 1980) as well as plasma membranes (N. Yamamoto & Kumashiro, 1993) and are ubiquitously expressed in circulating and tissue macrophages (Stamatos et al., 2005). Neu3 plays an important role in the brain where it desialylates G_{M3} ganglioside preventing its storage in lipofuscin bodies (Pan et al., 2017) but the enzyme has a broad substrate specificity and probably has other biological substrates outside the brain. No diseases are associated with Neu3 genetic deficiency in humans and *Neu3* KO mice are viable, fertile, grow normally and have normal life span (Seyrantepe, Canuel, et al., 2008; Smutova et al., 2014). Neu1 is active mostly against sialylated glycopeptides and oligosaccharides and is involved in their lysosomal catabolism (Carrillo, Milner, Ball, Snoek, & Campbell, 1997). Although complete genetic inactivation of *NEU1* in humans leads to severe neurological diseases, sialidosis and galactosialidosis (Pattison et al., 2004; Pshezhetsky et al., 1997; Ranganath et al., 2012; Seyrantepe et al., 2003) this does not rule out a possibility of Neu1 inhibition as a therapy. A perfect example is Angiotensin Converting

Enzyme (ACE): complete gene inactivation leads to renal tubular dysgenesis in humans and neonatal death (Gribouval et al., 2005). In spite of this, ACE inhibitors have probably played a more important role in reducing death from CAD in the last 30 years than any other medication. Indeed, our *Neu1* KI mouse model with 10% residual activity in tissues used in this study is not clinically affected (unless challenged by high-fat diet (Dridi et al., 2013)) whereas a complete *Neu1* KO develops a systemic disease matching the human phenotype (de Geest et al., 2002; X. Y. Zhou et al., 1995).

Our current data demonstrate that the genetic inactivation of both Neu1 and Neu3, but not Neu4 reduces the rate of atherosclerosis in the *ApoE* KO mice. In our opinion these results make neuraminidase 1 and 3 inhibitors potential candidates for treating atherosclerosis. We speculate, that it would be possible to find a dose threshold for partial pharmacological inhibition of neuraminidase 1 and 3 that has a preventive or therapeutic effect for atherogenesis without affecting catabolic pathways for sialoglycoconjugates.

4.3 Clinical implications and perspectives

It remains to be demonstrated whether effects of Neu1 and Neu3 inhibition translate into clinical efficacy for atherosclerosis and CAD. Optimal dosing and duration of treatment will have to be carefully evaluated in future preclinical work and clinical trials to ensure the best study design to demonstrate both safety and efficacy. In this regard, atherosclerosis is challenging because validated biomarkers mainly are based on lipids plasma composition and LDL levels in particular and do not take into account the atherogenic modification of LDL. In this respect, our findings on the involvement of neuraminidases in the pathology of atherosclerosis provide an

opportunity to develop new biomarkers based on LDL sialylation and neuraminidase activity in leucocytes.

4.4 Conclusions

Our experiments showed that mammalian neuraminidases are capable to discriminate between different sialic linkages, which may help to identify their biological targets. We also demonstrated that atherogenic derivatives of LDL can be produced by recombinant human neuraminidases 1 and 3. Using neuraminidase-deficient mouse models of atherosclerosis we have shown that the neuraminidase activity plays an essential role in triggering atherosclerosis suggesting that changes in sialylation can cause effects strong enough to influence predisposition to atherosclerosis in the human population.

Overall, our study suggests that strategies based on inhibition of leucocyte neuraminidases may have therapeutic potential for treating atherosclerosis. In particular, specific inhibitors of Neu1 and Neu3 or their derivatives could ultimately be used to test the viability of inhibitor-based therapy.

Chapter 5. References

Abdulkhalek, S., Amith, S. R., Franchuk, S. L., Jayanth, P., Guo, M., Finlay, T., . . . Szewczuk, M. R. (2011). Neu1 sialidase and matrix metalloproteinase-9 cross-talk is essential for Toll-like receptor activation and cellular signaling. *J Biol Chem, 286*(42), 36532-36549. doi:10.1074/jbc.M111.237578

Abifadel, M., Varret, M., Rabes, J. P., Allard, D., Ouguerram, K., Devillers, M., . . . Boileau, C. (2003). Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet, 34*(2), 154-156. doi:10.1038/ng1161

Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H., & Krieger, M. (1996). Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science, 271*(5248), 518-520.

Ahotupa, M., Suomela, J. P., Vuorimaa, T., & Vasankari, T. (2010). Lipoprotein-specific transport of circulating lipid peroxides. *Ann Med, 42*(7), 521-529. doi:10.3109/07853890.2010.510932

Albohy, A., Li, M. D., Zheng, R. B., Zou, C., & Cairo, C. W. (2010). Insight into substrate recognition and catalysis by the human neuraminidase 3 (NEU3) through molecular modeling and site-directed mutagenesis. *Glycobiology, 20*(9), 1127-1138. doi:10.1093/glycob/cwq077

Albohy, A., Mohan, S., Zheng, R. B., Pinto, B. M., & Cairo, C. W. (2011). Inhibitor selectivity of a new class of oseltamivir analogs against viral neuraminidase over human neuraminidase enzymes. *Bioorg Med Chem, 19*(9), 2817-2822. doi:10.1016/j.bmc.2011.03.039

AlHajri, L., AlHadhrami, A., AlMheiri, S., AlMutawa, Y., & AlHashimi, Z. (2017). The efficacy of evolocumab in the management of hyperlipidemia: a systematic review. *Ther Adv Cardiovasc Dis, 11*(5-6), 155-169. doi:10.1177/1753944717698925

Allain, C. C., Poon, L. S., Chan, C. S., Richmond, W., & Fu, P. C. (1974). Enzymatic determination of total serum cholesterol. *Clin Chem, 20*(4), 470-475.

Allende, M. L., & Proia, R. L. (2002). Lubricating cell signaling pathways with gangliosides. *Curr Opin Struct Biol, 12*(5), 587-592.

Aminoff, D., Bell, W. C., Fulton, I., & Ibgebrigtsen, N. (1976). Effect of sialidase on the viability of erythrocytes in circulation. *Am J Hematol, 1*(4), 419-432.

Amith, S. R., Jayanth, P., Franchuk, S., Siddiqui, S., Seyrantepe, V., Gee, K., . . . Szewczuk, M. R. (2009). Dependence of pathogen molecule-induced toll-like receptor activation and cell function on Neu1 sialidase. *Glycoconj J, 26*(9), 1197-1212. doi:10.1007/s10719-009-9239-8

Andrews, N. W. (2000). Regulated secretion of conventional lysosomes. *Trends Cell Biol, 10*(8), 316-321.

Arsenault, B. J., Kritikou, E. A., & Tardif, J. C. (2012). Regression of atherosclerosis. *Curr Cardiol Rep, 14*(4), 443-449. doi:10.1007/s11886-012-0285-7

Atkinson, D., Deckelbaum, R. J., Small, D. M., & Shipley, G. G. (1977). Structure of human plasma low-density lipoproteins: molecular organization of the central core. *Proc Natl Acad Sci U S A, 74*(3), 1042-1046.

Babal, P., Janega, P., Cerna, A., Kholova, I., & Brabencova, E. (2006). Neoplastic transformation of the thyroid gland is accompanied by changes in cellular sialylation. *Acta Histochem, 108*(2), 133-140. doi:10.1016/j.acthis.2006.03.003

Babelova, A., Sedding, D. G., & Brandes, R. P. (2013). Anti-atherosclerotic mechanisms of statin therapy. *Curr Opin Pharmacol, 13*(2), 260-264. doi:10.1016/j.coph.2013.01.004

Barter, P. J., Brandrup-Wognsen, G., Palmer, M. K., & Nicholls, S. J. (2010). Effect of statins on HDL-C: a complex process unrelated to changes in LDL-C: analysis of the VOYAGER Database. *J Lipid Res, 51*(6), 1546-1553. doi:10.1194/jlr.P002816

Bartlett, A. L., Grewal, T., De Angelis, E., Myers, S., & Stanley, K. K. (2000). Role of the macrophage galactose lectin in the uptake of desialylated LDL. *Atherosclerosis, 153*(1), 219-230.

Bartlett, A. L., & Stanley, K. K. (1998). All low density lipoprotein particles are partially desialylated in plasma. *Atherosclerosis, 138*(2), 237-245.

Basoglu, M., Yildirgan, M. I., Taysi, S., Yilmaz, I., Kiziltunc, A., Balik, A. A., . . . Atamanalp, S. S. (2003). Levels of soluble intercellular adhesion molecule-1 and total sialic acid in serum of patients with colorectal cancer. *J Surg Oncol, 83*(3), 180-184. doi:10.1002/jso.10257

Bentzon, J. F., Otsuka, F., Virmani, R., & Falk, E. (2014). Mechanisms of plaque formation and rupture. *Circ Res, 114*(12), 1852-1866. doi:10.1161/CIRCRESAHA.114.302721

Berbec, H., Paszkowska, A., Siwek, B., Gradziel, K., & Cybulski, M. (1999). Total serum sialic acid concentration as a supporting marker of malignancy in ovarian neoplasia. *Eur J Gynaecol Oncol, 20*(5-6), 389-392.

Berg, T., Gjoen, T., & Bakke, O. (1995). Physiological functions of endosomal proteolysis. *Biochem J, 307 (Pt 2)*, 313-326.

Bevan, A. P., Seabright, P. J., Tikerpae, J., Posner, B. I., Smith, G. D., & Siddle, K. (2000). The role of insulin dissociation from its endosomal receptor in insulin degradation. *Mol Cell Endocrinol, 164*(1-2), 145-157.

Bhakdi, S., Dorweiler, B., Kirchmann, R., Torzewski, J., Weise, E., Tranum-Jensen, J., . . . Wieland, E. (1995). On the pathogenesis of atherosclerosis: enzymatic transformation of human low density lipoprotein to an atherogenic moiety. *J Exp Med, 182*(6), 1959-1971.

Bigi, A., Morosi, L., Pozzi, C., Forcella, M., Tettamanti, G., Venerando, B., . . . Fusi, P. (2010). Human sialidase NEU4 long and short are extrinsic proteins bound to outer mitochondrial membrane and the endoplasmic reticulum, respectively. *Glycobiology, 20*(2), 148-157. doi:10.1093/glycob/cwp156

Boisvert, W. A., Spangenberg, J., & Curtiss, L. K. (1995). Treatment of severe hypercholesterolemia in apolipoprotein E-deficient mice by bone marrow transplantation. *J Clin Invest, 96*(2), 1118-1124. doi:10.1172/JCI118098

Bonten, E., van der Spoel, A., Fornerod, M., Grosveld, G., & d'Azzo, A. (1996). Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes Dev, 10*(24), 3156-3169.

Boring, L., Gosling, J., Cleary, M., & Charo, I. F. (1998). Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature, 394*(6696), 894-897. doi:10.1038/29788

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem, 72*, 248-254.

Brooks, S. A., & Leathem, A. J. (1998). Expression of N-acetyl galactosaminylated and sialylated glycans by metastases arising from primary breast cancer. *Invasion Metastasis, 18*(3), 115-121. doi:24504

Brown, M. S., Dana, S. E., & Goldstein, J. L. (1975). Cholesterol ester formation in cultured human fibroblasts. Stimulation by oxygenated sterols. *J Biol Chem, 250*(10), 4025-4027.

Brown, M. S., & Goldstein, J. L. (1979). Receptor-mediated endocytosis: insights from the lipoprotein receptor system. *Proc Natl Acad Sci U S A, 76*(7), 3330-3337.

Brown, M. S., & Goldstein, J. L. (1983). Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu Rev Biochem, 52*, 223-261. doi:10.1146/annurev.bi.52.070183.001255

Brown, M. S., & Goldstein, J. L. (1999). A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc Natl Acad Sci U S A, 96*(20), 11041-11048.

Bruce, C., Chouinard, R. A., Jr., & Tall, A. R. (1998). Plasma lipid transfer proteins, high-density lipoproteins, and reverse cholesterol transport. *Annu Rev Nutr, 18*, 297-330. doi:10.1146/annurev.nutr.18.1.297

Brunzell, J. D., Robertson, R. P., Lerner, R. L., Hazzard, W. R., Ensinck, J. W., Bierman, E. L., & Porte, D., Jr. (1976). Relationships between fasting plasma glucose levels and insulin secretion during intravenous glucose tolerance tests. *J Clin Endocrinol Metab, 42*(2), 222-229. doi:10.1210/jcem-42-2-222

Burmeister, W. P., Henrissat, B., Bosso, C., Cusack, S., & Ruigrok, R. W. (1993). Influenza B virus neuraminidase can synthesize its own inhibitor. *Structure, 1*(1), 19-26.

Byrne, B., Donohoe, G. G., & O'Kennedy, R. (2007). Sialic acids: carbohydrate moieties that influence the biological and physical properties of biopharmaceutical proteins and living cells. *Drug Discovery Today, 12*(7), 319-326.

Caligiuri, G., Nicoletti, A., Poirier, B., & Hansson, G. K. (2002). Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest, 109*(6), 745-753. doi:10.1172/JCI7272

Camejo, G., Fager, G., Rosengren, B., Hurt-Camejo, E., & Bondjers, G. (1993). Binding of low density lipoproteins by proteoglycans synthesized by proliferating and quiescent human arterial smooth muscle cells. *J Biol Chem, 268*(19), 14131-14137.

Camejo, G., Lopez, A., Lopez, F., & Quinones, J. (1985). Interaction of low density lipoproteins with arterial proteoglycans. The role of charge and sialic acid content. *Atherosclerosis, 55*(1), 93-105.

Carmena, R., Duriez, P., & Fruchart, J. C. (2004). Atherogenic lipoprotein particles in atherosclerosis. *Circulation, 109*(23 Suppl 1), III2-7. doi:10.1161/01.CIR.0000131511.50734.44

Carrillo, M. B., Milner, C. M., Ball, S. T., Snoek, M., & Campbell, R. D. (1997). Cloning and characterization of a sialidase from the murine histocompatibility-2 complex: low levels of mRNA and a single amino acid mutation are responsible for reduced sialidase activity in mice carrying the Neu1a allele. *Glycobiology, 7*(7), 975-986.

Charo, I. F., & Taub, R. (2011). Anti-inflammatory therapeutics for the treatment of atherosclerosis. *Nat Rev Drug Discov, 10*(5), 365-376. doi:10.1038/nrd3444

Chavas, L. M., Tringali, C., Fusi, P., Venerando, B., Tettamanti, G., Kato, R., . . . Wakatsuki, S. (2005). Crystal structure of the human cytosolic sialidase Neu2. Evidence for the dynamic

nature of substrate recognition. *J Biol Chem, 280*(1), 469-475. doi:10.1074/jbc.M411506200

Chen, X., & Varki, A. (2010). Advances in the biology and chemistry of sialic acids. *ACS Chem Biol, 5*(2), 163-176. doi:10.1021/cb900266r

Chen, X. P., Enioutina, E. Y., & Daynes, R. A. (1997). The control of IL-4 gene expression in activated murine T lymphocytes: a novel role for neu-1 sialidase. *J Immunol, 158*(7), 3070-3080.

Cherubini, A., Vigna, G. B., Zuliani, G., Ruggiero, C., Senin, U., & Fellin, R. (2005). Role of antioxidants in atherosclerosis: epidemiological and clinical update. *Curr Pharm Des, 11*(16), 2017-2032.

Chhatriwalla, A. K., Nicholls, S. J., Wang, T. H., Wolski, K., Sipahi, I., Crowe, T., . . . Nissen, S. E. (2009). Low levels of low-density lipoprotein cholesterol and blood pressure and progression of coronary atherosclerosis. *J Am Coll Cardiol, 53*(13), 1110-1115. doi:10.1016/j.jacc.2008.09.065

Chien, S. (1986). Blood rheology in myocardial infarction and hypertension. *Biorheology, 23*(6), 633-653.

Cohen, M., & Varki, A. (2010). The sialome--far more than the sum of its parts. *OMICS, 14*(4), 455-464. doi:10.1089/omi.2009.0148

Colletti, A., Derosa, G., & Cicero, A. F. (2016). Retargeting the management of hypercholesterolemia - focus on evolocumab. *Ther Clin Risk Manag, 12*, 1365-1376. doi:10.2147/TCRM.S116679

Comelli, E. M., Amado, M., Lustig, S. R., & Paulson, J. C. (2003). Identification and expression of Neu4, a novel murine sialidase. *Gene, 321*, 155-161.

Contreres, J. O., Faure, R., Baquiran, G., Bergeron, J. J., & Posner, B. I. (1998). ATP-dependent desensitization of insulin binding and tyrosine kinase activity of the insulin receptor kinase. The role of endosomal acidification. *J Biol Chem, 273*(34), 22007-22013.

Cross, A. S., Hyun, S. W., Miranda-Ribera, A., Feng, C., Liu, A., Nguyen, C., . . . Goldblum, S. E. (2012). NEU1 and NEU3 sialidase activity expressed in human lung microvascular endothelia: NEU1 restrains endothelial cell migration, whereas NEU3 does not. *J Biol Chem, 287*(19), 15966-15980. doi:10.1074/jbc.M112.346817

Cummings, R. D., Kornfeld, S., Schneider, W. J., Hobgood, K. K., Tolleshaug, H., Brown, M. S., & Goldstein, J. L. (1983). Biosynthesis of N- and O-linked oligosaccharides of the low density lipoprotein receptor. *J Biol Chem, 258*(24), 15261-15273.

Curtiss, L. K., & Boisvert, W. A. (2000). Apolipoprotein E and atherosclerosis. *Curr Opin Lipidol, 11*(3), 243-251.

Cybulsky, M. I., & Jongstra-Bilen, J. (2010). Resident intimal dendritic cells and the initiation of atherosclerosis. *Curr Opin Lipidol, 21*(5), 397-403. doi:10.1097/MOL.0b013e32833ded96

d'Azzo, A., Andria, G., Strisciuglio, G., & Galjaard, H. (1995). Galactosialidosis. In B. A. Scriver CR, Sly WS, Valle D. (Ed.), *Metabolic and Molecular Bases of Inherited Disease* (pp. 2835-2837). New Work, USA: McGraw-Hill.

Danehy, S. (2016). Pfizer discontinues global development of Bococizumab, its investigational PCSK9 inhibitor. From http://www.pfizer.com/news/press-release/press-release-detail/pfizer_discontinues_global_development_of_bococizumab_its_investigational_pcsk9_inhibitor.

Danesh, F. R., Anel, R. L., Zeng, L., Lomasney, J., Sahai, A., & Kanwar, Y. S. (2003). Immunomodulatory effects of HMG-CoA reductase inhibitors. *Arch Immunol Ther Exp (Warsz), 51*(3), 139-148.

Daugherty, A. (2002). Mouse models of atherosclerosis. *Am J Med Sci, 323*(1), 3-10.

Davies, P. F. (2000). Spatial hemodynamics, the endothelium, and focal atherogenesis: a cell cycle link? *Circ Res, 86*(2), 114-116.

de Geest, N., Bonten, E., Mann, L., de Sousa-Hitzler, J., Hahn, C., & d'Azzo, A. (2002). Systemic and neurologic abnormalities distinguish the lysosomal disorders sialidosis and galactosialidosis in mice. *Hum Mol Genet, 11*(12), 1455-1464.

de Lorgeril, M., & Salen, P. (2014). Do statins increase and Mediterranean diet decrease the risk of breast cancer? *BMC Med, 12*, 94. doi:10.1186/1741-7015-12-94

Dorland, L., Haverkamp, J., Viliegenthart, J. F., Strecker, G., Michalski, J. C., Fournet, B., . . . Montreuil, J. (1978). 360-MHz 1H nuclear-magnetic-resonance spectroscopy of sialyl-oligosaccharides from patients with sialidosis (mucolipidosis I and II). *Eur J Biochem, 87*(2), 323-329.

Drake, P. G., Balbis, A., Wu, J., Bergeron, J. J., & Posner, B. I. (2000). Association of phosphatidylinositol 3-kinase with the insulin receptor: compartmentation in rat liver. *Am J Physiol Endocrinol Metab, 279*(2), E266-274.

Dridi, L., Seyrantepe, V., Fougerat, A., Pan, X., Bonneil, E., Thibault, P., . . . Pshezhetsky, A. V. (2013). Positive regulation of insulin signaling by neuraminidase 1. *Diabetes, 62*(7), 2338-2346. doi:10.2337/db12-1825

Durand, S., Feldhammer, M., Bonneil, E., Thibault, P., & Pshezhetsky, A. V. (2010). Analysis of the biogenesis of heparan sulfate acetyl-CoA:alpha-glucosaminide N-acetyltransferase provides insights into the mechanism underlying its complete deficiency in mucopolysaccharidosis IIIC. *J Biol Chem, 285*(41), 31233-31242. doi:10.1074/jbc.M110.141150

Esser, V., Limbird, L. E., Brown, M. S., Goldstein, J. L., & Russell, D. W. (1988). Mutational analysis of the ligand binding domain of the low density lipoprotein receptor. *J Biol Chem, 263*(26), 13282-13290.

Esterbauer, H., Gebicki, J., Puhl, H., & Jurgens, G. (1992). The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic Biol Med, 13*(4), 341-390.

Fanzani, A., Giuliani, R., Colombo, F., Zizioli, D., Presta, M., Preti, A., & Marchesini, S. (2003). Overexpression of cytosolic sialidase Neu2 induces myoblast differentiation in C2C12 cells. *FEBS Lett, 547*(1-3), 183-188.

Fazio, S., & Linton, M. F. (2001). Mouse models of hyperlipidemia and atherosclerosis. *Front Biosci, 6*, D515-525.

Feijoo-Carnero, C., Rodriguez-Berrocal, F. J., Paez de la Cadena, M., Ayude, D., de Carlos, A., & Martinez-Zorzano, V. S. (2004). Clinical significance of preoperative serum sialic acid levels in colorectal cancer: utility in the detection of patients at high risk of tumor recurrence. *Int J Biol Markers, 19*(1), 38-45.

Feingold, K. R., & Grunfeld, C. (2012). Lipids: a key player in the battle between the host and microorganisms. *J Lipid Res, 53*(12), 2487-2489. doi:10.1194/jlr.E033407

Feng, C., Zhang, L., Almulki, L., Faez, S., Whitford, M., Hafezi-Moghadam, A., & Cross, A. S. (2011). Endogenous PMN sialidase activity exposes activation epitope on CD11b/CD18 which

enhances its binding interaction with ICAM-1. *J Leukoc Biol, 90*(2), 313-321. doi:10.1189/jlb.1210708

Fisher, W. R. (1972). The structure of the lower-density lipoproteins of human plasma: newer concepts derived from studies with the analytical ultracentrifuge. *Ann Clin Lab Sci, 2*(3), 198-208.

Fless, G. M., ZumMallen, M. E., & Scanu, A. M. (1986). Physicochemical properties of apolipoprotein(a) and lipoprotein(a-) derived from the dissociation of human plasma lipoprotein (a). *J Biol Chem, 261*(19), 8712-8718.

Flood, C., Gustafsson, M., Richardson, P. E., Harvey, S. C., Segrest, J. P., & Boren, J. (2002). Identification of the proteoglycan binding site in apolipoprotein B48. *J Biol Chem, 277*(35), 32228-32233. doi:10.1074/jbc.M204053200

Galjart, N. J., Morreau, H., Willemsen, R., Gillemans, N., Bonten, E. J., & d'Azzo, A. (1991). Human lysosomal protective protein has cathepsin A-like activity distinct from its protective function. *J Biol Chem, 266*(22), 14754-14762.

Gayral, S., Garnotel, R., Castaing-Berthou, A., Blaise, S., Fougerat, A., Berge, E., . . . Laffargue, M. (2014). Elastin-derived peptides potentiate atherosclerosis through the immune Neu1-PI3Kgamma pathway. *Cardiovasc Res, 102*(1), 118-127. doi:10.1093/cvr/cvt336

Getz, G. S., & Reardon, C. A. (2006). Diet and murine atherosclerosis. *Arterioscler Thromb Vasc Biol, 26*(2), 242-249. doi:10.1161/01.ATV.0000201071.49029.17

Giacopuzzi, E., Bresciani, R., Schauer, R., Monti, E., & Borsani, G. (2012). New insights on the sialidase protein family revealed by a phylogenetic analysis in metazoa. *PLoS One, 7*(8), e44193. doi:10.1371/journal.pone.0044193

Gimbrone, M. A., Jr., & Garcia-Cardena, G. (2013). Vascular endothelium, hemodynamics, and the pathobiology of atherosclerosis. *Cardiovasc Pathol, 22*(1), 9-15. doi:10.1016/j.carpath.2012.06.006

Goldberg, M. F., Cotlier, E., Fichenscher, L. G., Kenyon, K., Enat, R., & Borowsky, S. A. (1971). Macular cherry-red spot, corneal clouding, and beta-galactosidase deficiency. Clinical, biochemical, and electron microscopic study of a new autosomal recessive storage disease. *Arch Intern Med, 128*(3), 387-398.

Goldstein, J. L., Brown, M. S., Anderson, R. G., Russell, D. W., & Schneider, W. J. (1985). Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu Rev Cell Biol, 1*, 1-39. doi:10.1146/annurev.cb.01.110185.000245

Gorog, P., & Born, G. V. (1983). Uneven distribution of sialic acids on the luminal surface of aortic endothelium. *Br J Exp Pathol, 64*(4), 418-424.

Grewal, T., Bartlett, A., Burgess, J. W., Packer, N. H., & Stanley, K. K. (1996). Desialylated LDL uptake in human and mouse macrophages can be mediated by a lectin receptor. *Atherosclerosis, 121*(1), 151-163.

Gribouval, O., Gonzales, M., Neuhaus, T., Aziza, J., Bieth, E., Laurent, N., . . . Gubler, M. C. (2005). Mutations in genes in the renin-angiotensin system are associated with autosomal recessive renal tubular dysgenesis. *Nat Genet, 37*(9), 964-968. doi:10.1038/ng1623

Griffin, B. A. (1999). Lipoprotein atherogenicity: an overview of current mechanisms. *Proc Nutr Soc, 58*(1), 163-169.

Grundy, S. M. (1995). Role of low-density lipoproteins in atherogenesis and development of coronary heart disease. *Clin Chem, 41*(1), 139-146.

Grundy, S. M. (2002). Alternative approaches to cholesterol-lowering therapy. *Am J Cardiol, 90*(10), 1135-1138.

Hakala, J. K., Oksjoki, R., Laine, P., Du, H., Grabowski, G. A., Kovanen, P. T., & Pentikainen, M. O. (2003). Lysosomal enzymes are released from cultured human macrophages, hydrolyze LDL in vitro, and are present extracellularly in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol, 23*(8), 1430-1436. doi:10.1161/01.ATV.0000077207.49221.06

Hansson, G. K. (2005). Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med, 352*(16), 1685-1695. doi:10.1056/NEJMra043430

Harada, L. M., Carvalho, M. D., Passarelli, M., & Quintao, E. C. (1998). Lipoprotein desialylation simultaneously enhances the cell cholesterol uptake and impairs the reverse cholesterol transport system: in vitro evidences utilizing neuraminidase-treated lipoproteins and mouse peritoneal macrophages. *Atherosclerosis, 139*(1), 65-75.

Hasegawa, T., Sugeno, N., Takeda, A., Matsuzaki-Kobayashi, M., Kikuchi, A., Furukawa, K., . . . Itoyama, Y. (2007). Role of Neu4L sialidase and its substrate ganglioside GD3 in neuronal apoptosis induced by catechol metabolites. *FEBS Lett, 581*(3), 406-412. doi:10.1016/j.febslet.2006.12.046

Hast, J. (2003). Vessel wall biology. From: https://thoracickey.com/vessel-wall-biology/.

Hegele, R. A. (2009). Plasma lipoproteins: genetic influences and clinical implications. *Nat Rev Genet, 10*(2), 109-121. doi:10.1038/nrg2481

Heinecke, J. W. (1999). Mass spectrometric quantification of amino acid oxidation products in proteins: insights into pathways that promote LDL oxidation in the human artery wall. *FASEB J, 13*(10), 1113-1120.

Herve, F., Duche, J. C., & Jaurand, M. C. (1998). Changes in expression and microheterogeneity of the genetic variants of human alpha1-acid glycoprotein in malignant mesothelioma. *J Chromatogr B Biomed Sci Appl, 715*(1), 111-123.

Hoofnagle, A. N., & Heinecke, J. W. (2009). Lipoproteomics: using mass spectrometry-based proteomics to explore the assembly, structure, and function of lipoproteins. *J Lipid Res, 50*(10), 1967-1975. doi:10.1194/jlr.R900015-JLR200

Hoover-Plow, J., & Huang, M. (2013). Lipoprotein(a) metabolism: potential sites for therapeutic targets. *Metabolism, 62*(4), 479-491. doi:10.1016/j.metabol.2012.07.024

Hopkins, P. N. (2013). Molecular biology of atherosclerosis. *Physiol Rev, 93*(3), 1317-1542. doi:10.1152/physrev.00004.2012

Huff, M. W., Pollex, R. L., & Hegele, R. A. (2006). NPC1L1: evolution from pharmacological target to physiological sterol transporter. *Arterioscler Thromb Vasc Biol, 26*(11), 2433-2438. doi:10.1161/01.ATV.0000245791.53245.ee

Iijima, S., Shiba, K., Kimura, M., Nagai, K., & Iwai, T. (2000). Changes of alpha1-acid glycoprotein microheterogeneity in acute inflammation stages analyzed by isoelectric focusing using serum obtained postoperatively. *Electrophoresis, 21*(4), 753-759. doi:10.1002/(SICI)1522-2683(20000301)21:4<753::AID-ELPS753>3.0.CO;2-Y

Ishibashi, S., Goldstein, J. L., Brown, M. S., Herz, J., & Burns, D. K. (1994). Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J Clin Invest, 93*(5), 1885-1893. doi:10.1172/JCI117179

Ishii, H., Kobayashi, M., Kurebayashi, N., Yoshikawa, D., Suzuki, S., Ichimiya, S., . . . Murohara, T. (2013). Impact of angiotensin II receptor blocker therapy (olmesartan or valsartan) on

coronary atherosclerotic plaque volume measured by intravascular ultrasound in patients with stable angina pectoris. *Am J Cardiol, 112*(3), 363-368. doi:10.1016/j.amjcard.2013.03.038

Ismail, N. A., Alavi, M. Z., & Moore, S. (1994). Lipoprotein-proteoglycan complexes from injured rabbit aortas accelerate lipoprotein uptake by arterial smooth muscle cells. *Atherosclerosis, 105*(1), 79-87.

Janeway, C. A., Jr., & Medzhitov, R. (2002). Innate immune recognition. *Annu Rev Immunol, 20*, 197-216. doi:10.1146/annurev.immunol.20.083001.084359

Jawien, J., Nastalek, P., & Korbut, R. (2004). Mouse models of experimental atherosclerosis. *J Physiol Pharmacol, 55*(3), 503-517.

Jones, C. J., Aplin, J. D., Mulholland, J., & Glasser, S. R. (1993). Patterns of sialylation in differentiating rat decidual cells as revealed by lectin histochemistry. *J Reprod Fertil, 99*(2), 635-645.

Jones, G. T., van Rij, A. M., Cole, J., Williams, M. J., Bateman, E. H., Marcovina, S. M., . . . McCormick, S. P. (2007). Plasma lipoprotein(a) indicates risk for 4 distinct forms of vascular disease. *Clin Chem, 53*(4), 679-685. doi:10.1373/clinchem.2006.079947

Kahn, S. E. (2000). The importance of the beta-cell in the pathogenesis of type 2 diabetes mellitus. *Am J Med, 108 Suppl 6a*, 2S-8S.

Kakino, A., Fujita, Y., Nakano, A., Horiuchi, S., & Sawamura, T. (2016). Developmental Endothelial Locus-1 (Del-1) Inhibits Oxidized Low-Density Lipoprotein Activity by Direct Binding, and Its Overexpression Attenuates Atherogenesis in Mice. *Circ J*. doi:10.1253/circj.CJ-16-0808

Kalanuria, A. A., Nyquist, P., & Ling, G. (2012). The prevention and regression of atherosclerotic plaques: emerging treatments. *Vasc Health Risk Manag, 8*, 549-561. doi:10.2147/VHRM.S27764

Kalin, M. F., & Zumoff, B. (1990). Sex hormones and coronary disease: a review of the clinical studies. *Steroids, 55*(8), 330-352.

Kane, J. P. (1983). Apolipoprotein B: structural and metabolic heterogeneity. *Annu Rev Physiol, 45*, 637-650. doi:10.1146/annurev.ph.45.030183.003225

Kearney, P. M., Baigent, C., Godwin, J., Halls, H., Emberson, J. R., & Patrono, C. (2006). Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. *BMJ, 332*(7553), 1302-1308. doi:10.1136/bmj.332.7553.1302

Kelm, S., & Schauer, R. (1997). Sialic acids in molecular and cellular interactions. *Int Rev Cytol, 175*, 137-240.

Kerzner, B., Corbelli, J., Sharp, S., Lipka, L. J., Melani, L., LeBeaut, A., . . . Ezetimibe Study, G. (2003). Efficacy and safety of ezetimibe coadministered with lovastatin in primary hypercholesterolemia. *Am J Cardiol, 91*(4), 418-424.

Keys, A. (1997). Coronary heart disease in seven countries. 1970. *Nutrition, 13*(3), 250-252; discussion 249, 253.

Khedri, Z., Muthana, M. M., Li, Y., Muthana, S. M., Yu, H., Cao, H., & Chen, X. (2012). Probe sialidase substrate specificity using chemoenzymatically synthesized sialosides containing C9-modified sialic acid. *Chem Commun (Camb), 48*(27), 3357-3359. doi:10.1039/c2cc17393j

Kirschner, K. N., Yongye, A. B., Tschampel, S. M., Gonzalez-Outeirino, J., Daniels, C. R., Foley, B. L., & Woods, R. J. (2008). GLYCAM06: A generalizable Biomolecular force field. Carbohydrates. *Journal of Computational Chemistry, 29*(4), 622-655. doi:10.1002/jcc.20820

Kjolby, M., Nielsen, M. S., & Petersen, C. M. (2015). Sortilin, encoded by the cardiovascular risk gene SORT1, and its suggested functions in cardiovascular disease. *Curr Atheroscler Rep, 17*(4), 496. doi:10.1007/s11883-015-0496-7

Knibbs, R. N., Goldstein, I. J., Ratcliffe, R. M., & Shibuya, N. (1991). Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from Maackia amurensis. Comparison with other sialic acid-specific lectins. *J Biol Chem, 266*(1), 83-88.

Knuiman, M. W., Watts, G. F., & Divitini, M. L. (2004). Is sialic acid an independent risk factor for cardiovascular disease? A 17-year follow-up study in Busselton, Western Australia. *Ann Epidemiol, 14*(9), 627-632. doi:10.1016/j.annepidem.2003.09.017

Koda, T., Kijimoto-Ochiai, S., Uemura, S., & Inokuchi, J. (2009). Specific expression of Neu2 type B in mouse thymus and the existence of a membrane-bound form in COS cells. *Biochem Biophys Res Commun, 387*(4), 729-735. doi:10.1016/j.bbrc.2009.07.100

Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P., & Krieger, M. (1990). Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature, 343*(6258), 531-535. doi:10.1038/343531a0

Koenen, R. R., & Weber, C. (2010). Therapeutic targeting of chemokine interactions in atherosclerosis. *Nat Rev Drug Discov, 9*(2), 141-153. doi:10.1038/nrd3048

Koseki, K., Wada, T., Hosono, M., Hata, K., Yamaguchi, K., Nitta, K., & Miyagi, T. (2012). Human cytosolic sialidase NEU2-low general tissue expression but involvement in PC-3 prostate cancer cell survival. *Biochem Biophys Res Commun, 428*(1), 142-149. doi:10.1016/j.bbrc.2012.10.028

Kotani, K., Kuroiwa, A., Saito, T., Matsuda, Y., Koda, T., & Kijimoto-Ochiai, S. (2001). Cloning, chromosomal mapping, and characteristic 5'-UTR sequence of murine cytosolic sialidase. *Biochem Biophys Res Commun, 286*(2), 250-258. doi:10.1006/bbrc.2001.5374

Krauss, R. M. (1995). Dense low density lipoproteins and coronary artery disease. *Am J Cardiol, 75*(6), 53B-57B.

Kwan, B. C., Kronenberg, F., Beddhu, S., & Cheung, A. K. (2007). Lipoprotein metabolism and lipid management in chronic kidney disease. *J Am Soc Nephrol, 18*(4), 1246-1261. doi:10.1681/ASN.2006091006

Lambre, C. R., Greffard, A., Gattegno, L., & Saffar, L. (1990). Modifications of sialidase activity during the monocyte-macrophage differentiation in vitro. *Immunol Lett, 23*(3), 179-182.

Lamon-Fava, S. (2013). Statins and lipid metabolism: an update. *Curr Opin Lipidol, 24*(3), 221-226. doi:10.1097/MOL.0b013e3283613b8b

Larsen, S., Stride, N., Hey-Mogensen, M., Hansen, C. N., Bang, L. E., Bundgaard, H., . . . Dela, F. (2013). Simvastatin effects on skeletal muscle: relation to decreased mitochondrial function and glucose intolerance. *J Am Coll Cardiol, 61*(1), 44-53. doi:10.1016/j.jacc.2012.09.036

Law, M. R., Wald, N. J., & Thompson, S. G. (1994). By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease? *BMJ, 308*(6925), 367-372.

Lee, Y. C., Townsend, R. R., Hardy, M. R., Lonngren, J., Arnarp, J., Haraldsson, M., & Lonn, H. (1983). Binding of synthetic oligosaccharides to the hepatic Gal/GalNAc lectin. Dependence on fine structural features. *J Biol Chem, 258*(1), 199-202.

Lehmann, F., Tiralongo, E., & Tiralongo, J. (2006). Sialic acid-specific lectins: occurrence, specificity and function. *Cell Mol Life Sci, 63*(12), 1331-1354. doi:10.1007/s00018-005-5589-y

Lepor, N. E., & Kereiakes, D. J. (2015). The PCSK9 Inhibitors: A Novel Therapeutic Target Enters Clinical Practice. *Am Health Drug Benefits, 8*(9), 483-489.

Levitan, I., Volkov, S., & Subbaiah, P. V. (2010). Oxidized LDL: diversity, patterns of recognition, and pathophysiology. *Antioxid Redox Signal, 13*(1), 39-75. doi:10.1089/ars.2009.2733

Levy, E., Thibault, L., Roy, C. C., Letarte, J., Lambert, M., & Seidman, E. G. (1990). Mechanisms of hypercholesterolaemia in glycogen storage disease type I: defective metabolism of low density lipoprotein in cultured skin fibroblasts. *Eur J Clin Invest, 20*(3), 253-260.

Lewis, B. (1973). Classification of lipoproteins and lipoprotein disorders. *J Clin Pathol Suppl (Assoc Clin Pathol), 5*, 26-31.

Liang, F., Seyrantepe, V., Landry, K., Ahmad, R., Ahmad, A., Stamatos, N. M., & Pshezhetsky, A. V. (2006). Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. *J Biol Chem, 281*(37), 27526-27538. doi:10.1074/jbc.M605633200

Libby, P. (2002). Inflammation in atherosclerosis. *Nature, 420*(6917), 868-874. doi:10.1038/nature01323

Libby, P., Ridker, P. M., Hansson, G. K., & Leducq Transatlantic Network on, A. (2009). Inflammation in atherosclerosis: from pathophysiology to practice. *J Am Coll Cardiol, 54*(23), 2129-2138. doi:10.1016/j.jacc.2009.09.009

Libby, P., Ridker, P. M., & Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation, 105*(9), 1135-1143.

Lindberg, G. (2007). Resialylation of sialic acid deficit vascular endothelium, circulating cells and macromolecules may counteract the development of atherosclerosis: a hypothesis. *Atherosclerosis, 192*(2), 243-245. doi:10.1016/j.atherosclerosis.2007.03.011

Linton, M. F., Yancey, P. G., Davies, S. S., Jerome, W. G. J., Linton, E. F., & Vickers, K. C. (2000). The Role of Lipids and Lipoproteins in Atherosclerosis. In L. J. De Groot, G. Chrousos, K. Dungan, K. R. Feingold, A. Grossman, J. M. Hershman, C. Koch, M. Korbonits, R. McLachlan, M. New, J. Purnell, R. Rebar, F. Singer, & A. Vinik (Eds.), *Endotext*. South Dartmouth (MA).

Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., . . . Memish, Z. A. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet, 380*(9859), 2095-2128. doi:10.1016/S0140-6736(12)61728-0

Ludewig, B., Freigang, S., Jaggi, M., Kurrer, M. O., Pei, Y. C., Vlk, L., . . . Hengartner, H. (2000). Linking immune-mediated arterial inflammation and cholesterol-induced atherosclerosis in a transgenic mouse model. *Proc Natl Acad Sci U S A, 97*(23), 12752-12757. doi:10.1073/pnas.220427097

Lukong, K. E., Seyrantepe, V., Landry, K., Trudel, S., Ahmad, A., Gahl, W. A., . . . Pshezhetsky, A. V. (2001). Intracellular distribution of lysosomal sialidase is controlled by the internalization signal in its cytoplasmic tail. *J Biol Chem, 276*(49), 46172-46181. doi:10.1074/jbc.M104547200

Lusis, A. J. (2000). Atherosclerosis. *Nature, 407*(6801), 233-241. doi:10.1038/35025203

Macri, J., & Adeli, K. (1997). Conformational changes in apolipoprotein B modulate intracellular assembly and degradation of ApoB-containing lipoprotein particles in HepG2 cells. *Arterioscler Thromb Vasc Biol, 17*(11), 2982-2994.

Mallat, Z., Taleb, S., Ait-Oufella, H., & Tedgui, A. (2009). The role of adaptive T cell immunity in atherosclerosis. *J Lipid Res, 50 Suppl*, S364-369. doi:10.1194/jlr.R800092-JLR200

Mallika, V., Goswami, B., & Rajappa, M. (2007). Atherosclerosis pathophysiology and the role of novel risk factors: a clinicobiochemical perspective. *Angiology, 58*(5), 513-522. doi:10.1177/0003319707303443

Martin, M. J., Hulley, S. B., Browner, W. S., Kuller, L. H., & Wentworth, D. (1986). Serum cholesterol, blood pressure, and mortality: implications from a cohort of 361,662 men. *Lancet, 2*(8513), 933-936.

Mauger, J. F., Couture, P., Bergeron, N., & Lamarche, B. (2006). Apolipoprotein C-III isoforms: kinetics and relative implication in lipid metabolism. *J Lipid Res, 47*(6), 1212-1218. doi:10.1194/jlr.M500455-JLR200

McCloy, R. A., Rogers, S., Caldon, C. E., Lorca, T., Castro, A., & Burgess, A. (2014). Partial inhibition of Cdk1 in G 2 phase overrides the SAC and decouples mitotic events. *Cell Cycle, 13*(9), 1400-1412. doi:10.4161/cc.28401

Meager, A. (1999). Cytokine regulation of cellular adhesion molecule expression in inflammation. *Cytokine Growth Factor Rev, 10*(1), 27-39.

Meir, K. S., & Leitersdorf, E. (2004). Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. *Arterioscler Thromb Vasc Biol, 24*(6), 1006-1014. doi:10.1161/01.ATV.0000128849.12617.f4

Mel'nichenko, A. A., Tertov, V. V., Ivanova, O. A., Aksenov, D. V., Sobenin, I. A., Popov, E. V., . . . Orekhov, A. N. (2005). Desialylation decreases the resistance of apo B-containing lipoproteins to aggregation and increases their atherogenic potential. *Bull Exp Biol Med, 140*(1), 51-54.

Meng, C. Q. (2002). Ezetimibe. Schering-Plough. *Curr Opin Investig Drugs, 3*(3), 427-432.

Meyrelles, S. S., Peotta, V. A., Pereira, T. M., & Vasquez, E. C. (2011). Endothelial dysfunction in the apolipoprotein E-deficient mouse: insights into the influence of diet, gender and aging. *Lipids Health Dis, 10*, 211. doi:10.1186/1476-511X-10-211

Michalski, J. C., Strecker, G., & Fournet, B. (1977). Structures of sialyl-oligosaccharides excreted in the urine of a patient with mucolipidosis I. *FEBS Lett, 79*(1), 101-104.

Millar, J. S. (2001). The sialylation of plasma lipoproteins. *Atherosclerosis, 154*(1), 1-13.

Milner, C. M., Smith, S. V., Carrillo, M. B., Taylor, G. L., Hollinshead, M., & Campbell, R. D. (1997). Identification of a sialidase encoded in the human major histocompatibility complex. *J Biol Chem, 272*(7), 4549-4558.

Miyagi, T., Konno, K., Emori, Y., Kawasaki, H., Suzuki, K., Yasui, A., & Tsuik, S. (1993). Molecular cloning and expression of cDNA encoding rat skeletal muscle cytosolic sialidase. *J Biol Chem, 268*(35), 26435-26440.

Miyagi, T., & Tsuiki, S. (1985). Purification and characterization of cytosolic sialidase from rat liver. *J Biol Chem, 260*(11), 6710-6716.

Miyagi, T., & Yamaguchi, K. (2012). Mammalian sialidases: physiological and pathological roles in cellular functions. *Glycobiology, 22*(7), 880-896. doi:10.1093/glycob/cws057

Mochalova, L., Kurova, V., Shtyrya, Y., Korchagina, E., Gambaryan, A., Belyanchikov, I., & Bovin, N. (2007). Oligosaccharide specificity of influenza H1N1 virus neuraminidases. *Arch Virol, 152*(11), 2047-2057. doi:10.1007/s00705-007-1024-z

Monti, E., Bassi, M. T., Bresciani, R., Civini, S., Croci, G. L., Papini, N., . . . Borsani, G. (2004). Molecular cloning and characterization of NEU4, the fourth member of the human sialidase gene family. *Genomics, 83*(3), 445-453. doi:10.1016/j.ygeno.2003.08.019

Monti, E., Bassi, M. T., Papini, N., Riboni, M., Manzoni, M., Venerando, B., . . . Borsani, G. (2000). Identification and expression of NEU3, a novel human sialidase associated to the plasma membrane. *Biochem J, 349*(Pt 1), 343-351.

Monti, E., Preti, A., Nesti, C., Ballabio, A., & Borsani, G. (1999). Expression of a novel human sialidase encoded by the NEU2 gene. *Glycobiology, 9*(12), 1313-1321.

Monti, E., Preti, A., Rossi, E., Ballabio, A., & Borsani, G. (1999). Cloning and characterization of NEU2, a human gene homologous to rodent soluble sialidases. *Genomics, 57*(1), 137-143. doi:10.1006/geno.1999.5749

Moore, K. J., & Freeman, M. W. (2006). Scavenger receptors in atherosclerosis: beyond lipid uptake. *Arterioscler Thromb Vasc Biol, 26*(8), 1702-1711. doi:10.1161/01.ATV.0000229218.97976.43

Morita, S. Y. (2016). Metabolism and Modification of Apolipoprotein B-Containing Lipoproteins Involved in Dyslipidemia and Atherosclerosis. *Biol Pharm Bull, 39*(1), 1-24. doi:10.1248/bpb.b15-00716

Morrissey, R. P., Diamond, G. A., & Kaul, S. (2009). Statins in acute coronary syndromes: do the guideline recommendations match the evidence? *J Am Coll Cardiol, 54*(15), 1425-1433. doi:10.1016/j.jacc.2009.04.093

Musliner, T. A., McVicker, K. M., Iosefa, J. F., & Krauss, R. M. (1987). Lipolysis products promote the formation of complexes of very-low-density and low-density lipoproteins. *Biochim Biophys Acta, 919*(2), 97-110.

Nakashima, Y., Plump, A. S., Raines, E. W., Breslow, J. L., & Ross, R. (1994). ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb, 14*(1), 133-140.

Nakashima, Y., Raines, E. W., Plump, A. S., Breslow, J. L., & Ross, R. (1998). Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arterioscler Thromb Vasc Biol, 18*(5), 842-851.

Napoli, C., D'Armiento, F. P., Mancini, F. P., Postiglione, A., Witztum, J. L., Palumbo, G., & Palinski, W. (1997). Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. *J Clin Invest, 100*(11), 2680-2690. doi:10.1172/JCI119813

Natori, Y., Ohkura, N., Nasui, M., Atsumi, G., & Kihara-Negishi, F. (2013). Acidic sialidase activity is aberrant in obese and diabetic mice. *Biol Pharm Bull, 36*(6), 1027-1031.

Nievelstein, P. F., Fogelman, A. M., Mottino, G., & Frank, J. S. (1991). Lipid accumulation in rabbit aortic intima 2 hours after bolus infusion of low density lipoprotein. A deep-etch and immunolocalization study of ultrarapidly frozen tissue. *Arterioscler Thromb, 11*(6), 1795-1805.

Noble, R. P. (1968). Electrophoretic separation of plasma lipoproteins in agarose gel. *J Lipid Res, 9*(6), 693-700.

Nozue, T., Hattori, H., Ogawa, K., Kujiraoka, T., Iwasaki, T., & Michishita, I. (2016). Effects of Statin Therapy on Plasma Proprotein Convertase Subtilisin/kexin Type 9 and Sortilin Levels in Statin-Naive Patients with Coronary Artery Disease. *J Atheroscler Thromb, 23*(7), 848-856. doi:10.5551/jat.33407

Okamura-Oho, Y., Zhang, S., & Callahan, J. W. (1994). The biochemistry and clinical features of galactosialidosis. *Biochim Biophys Acta, 1225*(3), 244-254.

Okuyama, H., Langsjoen, P. H., Hamazaki, T., Ogushi, Y., Hama, R., Kobayashi, T., & Uchino, H. (2015). Statins stimulate atherosclerosis and heart failure: pharmacological mechanisms. *Expert Rev Clin Pharmacol, 8*(2), 189-199. doi:10.1586/17512433.2015.1011125

Orekhov, A. N., Tertov, V. V., & Mukhin, D. N. (1991). Desialylated low density lipoprotein--naturally occurring modified lipoprotein with atherogenic potency. *Atherosclerosis, 86*(2-3), 153-161.

Orekhov, A. N., Tertov, V. V., Sobenin, I. A., Smirnov, V. N., Via, D. P., Guevara, J., Jr., . . . Morrisett, J. D. (1992). Sialic acid content of human low density lipoproteins affects their interaction with cell receptors and intracellular lipid accumulation. *J Lipid Res, 33*(6), 805-817.

Packard, R. R., & Libby, P. (2008). Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clin Chem, 54*(1), 24-38. doi:10.1373/clinchem.2007.097360

Paigen, B., Morrow, A., Brandon, C., Mitchell, D., & Holmes, P. (1985). Variation in susceptibility to atherosclerosis among inbred strains of mice. *Atherosclerosis, 57*(1), 65-73.

Pan, X., De Aragao, C. B. P., Velasco-Martin, J. P., Priestman, D. A., Wu, H. Y., Takahashi, K., . . . Pshezhetsky, A. V. (2017). Neuraminidases 3 and 4 regulate neuronal function by catabolizing brain gangliosides. *FASEB J*. doi:10.1096/fj.201601299R

Patrono, C., Coller, B., FitzGerald, G. A., Hirsh, J., & Roth, G. (2004). Platelet-active drugs: the relationships among dose, effectiveness, and side effects: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. *Chest, 126*(3 Suppl), 234S-264S. doi:10.1378/chest.126.3_suppl.234S

Pattison, S., Pankarican, M., Rupar, C. A., Graham, F. L., & Igdoura, S. A. (2004). Five novel mutations in the lysosomal sialidase gene (NEU1) in type II sialidosis patients and assessment of their impact on enzyme activity and intracellular targeting using adenovirus-mediated expression. *Hum Mutat, 23*(1), 32-39. doi:10.1002/humu.10278

Pazynina, G., Tuzikov, A., Chinarev, A., Obukhova, P., & Bovin, N. (2002). Simple stereoselective synthesis of α2-6 sialooligosaccharides. *Tetrahedron Letters, 43*(45), 8011-8013. doi:http://dx.doi.org/10.1016/S0040-4039(02)01983-4

Pepin, J. M., O'Neil, J. A., & Hoff, H. F. (1991). Quantification of apo[a] and apoB in human atherosclerotic lesions. *J Lipid Res, 32*(2), 317-327.

Perrey, S., Ishibashi, S., Kitamine, T., Osuga, J., Yagyu, H., Chen, Z., . . . Yamada, N. (2001). The LDL receptor is the major pathway for beta-VLDL uptake by mouse peritoneal macrophages. *Atherosclerosis, 154*(1), 51-60.

Pilatte, Y., Bignon, J., & Lambre, C. R. (1993). Sialic acids as important molecules in the regulation of the immune system: pathophysiological implications of sialidases in immunity. *Glycobiology, 3*(3), 201-218.

Pitas, R. E., Innerarity, T. L., Weinstein, J. N., & Mahley, R. W. (1981). Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. *Arteriosclerosis, 1*(3), 177-185.

Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setala, K., Walsh, A., Verstuyft, J. G., . . . Breslow, J. L. (1992). Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell, 71*(2), 343-353.

Pshezhetsky, A. V., & Ashmarina, L. I. (2013). Desialylation of surface receptors as a new dimension in cell signaling. *Biochemistry (Mosc), 78*(7), 736-745. doi:10.1134/S0006297913070067

Pshezhetsky, A. V., & Ashmarina, M. (2001). Lysosomal multienzyme complex: biochemistry, genetics, and molecular pathophysiology. *Prog Nucleic Acid Res Mol Biol, 69*, 81-114.

Pshezhetsky, A. V., & Hinek, A. (2011). Where catabolism meets signalling: neuraminidase 1 as a modulator of cell receptors. *Glycoconj J, 28*(7), 441-452. doi:10.1007/s10719-011-9350-5

Pshezhetsky, A. V., & Potier, M. (1996). Association of N-acetylgalactosamine-6-sulfate sulfatase with the multienzyme lysosomal complex of beta-galactosidase, cathepsin A, and neuraminidase. Possible implication for intralysosomal catabolism of keratan sulfate. *J Biol Chem, 271*(45), 28359-28365.

Pshezhetsky, A. V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M. A., . . . Potier, M. (1997). Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nat Genet, 15*(3), 316-320. doi:10.1038/ng0397-316

Rader, D. J., & Daugherty, A. (2008). Translating molecular discoveries into new therapies for atherosclerosis. *Nature, 451*(7181), 904-913. doi:10.1038/nature06796

Rajpura, K. B., Patel, P. S., Chawda, J. G., & Shah, R. M. (2005). Clinical significance of total and lipid bound sialic acid levels in oral pre-cancerous conditions and oral cancer. *J Oral Pathol Med, 34*(5), 263-267. doi:10.1111/j.1600-0714.2004.00210.x

Ranganath, P., Sharma, V., Danda, S., Nandineni, M. R., & Dalal, A. B. (2012). Novel mutations in the neuraminidase-1 (NEU1) gene in two patients of sialidosis in India. *Indian J Med Res, 136*(6), 1048-1050.

Ray, K. K., Cannon, C. P., & Braunwald, E. (2007). Recent trials of lipid lowering. *Int J Clin Pract, 61*(7), 1145-1159. doi:10.1111/j.1742-1241.2007.01425.x

Reardon, C. A., & Getz, G. S. (2001). Mouse models of atherosclerosis. *Curr Opin Lipidol, 12*(2), 167-173.

Regina Todeschini, A., & Hakomori, S. I. (2008). Functional role of glycosphingolipids and gangliosides in control of cell adhesion, motility, and growth, through glycosynaptic microdomains. *Biochim Biophys Acta, 1780*(3), 421-433. doi:10.1016/j.bbagen.2007.10.008

Rocha, V. Z., & Libby, P. (2009). Obesity, inflammation, and atherosclerosis. *Nat Rev Cardiol, 6*(6), 399-409. doi:10.1038/nrcardio.2009.55

Roeschlau, P., Bernt, E., & Gruber, W. (1974). Enzymatic determination of total cholesterol in serum. *Z Klin Chem Klin Biochem, 12*(5), 226.

Romppanen, J., Haapalainen, T., Punnonen, K., & Penttila, I. (2002). Serum sialic acid and prostate-specific antigen in differential diagnosis of benign prostate hyperplasia and prostate cancer. *Anticancer Res, 22*(1A), 415-420.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature, 362*(6423), 801-809. doi:10.1038/362801a0

Ross, R. (1999). Atherosclerosis is an inflammatory disease. *Am Heart J, 138*(5 Pt 2), S419-420.

Roy, S. (2014). Atherosclerotic Cardiovascular Disease Risk and Evidence-based Management of Cholesterol. *N Am J Med Sci, 6*(5), 191-198. doi:10.4103/1947-2714.132916

Ruelland, A., Gallou, G., Legras, B., Paillard, F., & Cloarec, L. (1993). LDL sialic acid content in patients with coronary artery disease. *Clin Chim Acta, 221*(1-2), 127-133.

Sabarinath, P. S., & Appukuttan, P. S. (2015). Immunopathology of desialylation: human plasma lipoprotein(a) and circulating anti-carbohydrate antibodies form immune complexes that recognize host cells. *Mol Cell Biochem, 403*(1-2), 13-23. doi:10.1007/s11010-015-2332-3

Sandbhor, M. S., Soya, N., Albohy, A., Zheng, R. B., Cartmell, J., Bundle, D. R., . . . Cairo, C. W. (2011). Substrate recognition of the membrane-associated sialidase NEU3 requires a hydrophobic aglycone. *Biochemistry, 50*(32), 6753-6762. doi:10.1021/bi200449j

Saremi, A., Bahn, G., Reaven, P. D., & Investigators, V. (2012). Progression of vascular calcification is increased with statin use in the Veterans Affairs Diabetes Trial (VADT). *Diabetes Care, 35*(11), 2390-2392. doi:10.2337/dc12-0464

Sato, K., & Miyagi, T. (1996). Involvement of an endogenous sialidase in skeletal muscle cell differentiation. *Biochem Biophys Res Commun, 221*(3), 826-830. doi:10.1006/bbrc.1996.0681

Sawayama, Y., Shimizu, C., Maeda, N., Tatsukawa, M., Kinukawa, N., Koyanagi, S., . . . Hayashi, J. (2002). Effects of probucol and pravastatin on common carotid atherosclerosis in patients with asymptomatic hypercholesterolemia. Fukuoka Atherosclerosis Trial (FAST). *J Am Coll Cardiol, 39*(4), 610-616.

Scanu, A. M., Lawn, R. M., & Berg, K. (1991). Lipoprotein(a) and atherosclerosis. *Ann Intern Med, 115*(3), 209-218.

Schaefer, E. J., Gregg, R. E., Ghiselli, G., Forte, T. M., Ordovas, J. M., Zech, L. A., & Brewer, H. B., Jr. (1986). Familial apolipoprotein E deficiency. *J Clin Invest, 78*(5), 1206-1219. doi:10.1172/JCI112704

Schmidli, R. (2016). PCSK9 inhibitors - clinical applications. *Aust Prescr, 39*(5), 168-170. doi:10.18773/austprescr.2016.061

Schonfeld, G., & Krul, E. S. (1986). Immunologic approaches to lipoprotein structure. *J Lipid Res, 27*(6), 583-601.

Schumaker, V. N., Phillips, M. L., & Chatterton, J. E. (1994). Apolipoprotein B and low-density lipoprotein structure: implications for biosynthesis of triglyceride-rich lipoproteins. *Adv Protein Chem, 45*, 205-248.

Schwartz, C. J., Valente, A. J., Sprague, E. A., Kelley, J. L., & Nerem, R. M. (1991). The pathogenesis of atherosclerosis: an overview. *Clin Cardiol, 14*(2 Suppl 1), I1-16.

Segrest, J. P., Jones, M. K., De Loof, H., & Dashti, N. (2001). Structure of apolipoprotein B-100 in low density lipoproteins. *J Lipid Res, 42*(9), 1346-1367.

Seidah, N. G., Benjannet, S., Wickham, L., Marcinkiewicz, J., Jasmin, S. B., Stifani, S., . . . Chretien, M. (2003). The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. *Proc Natl Acad Sci U S A, 100*(3), 928-933. doi:10.1073/pnas.0335507100

Seyrantepe, V., Canuel, M., Carpentier, S., Landry, K., Durand, S., Liang, F., . . . Pshezhetsky, A. V. (2008). Mice deficient in Neu4 sialidase exhibit abnormal ganglioside catabolism and lysosomal storage. *Hum Mol Genet, 17*(11), 1556-1568. doi:10.1093/hmg/ddn043

Seyrantepe, V., Hinek, A., Peng, J., Fedjaev, M., Ernest, S., Kadota, Y., . . . Pshezhetsky, A. V. (2008). Enzymatic activity of lysosomal carboxypeptidase (cathepsin) A is required for proper elastic fiber formation and inactivation of endothelin-1. *Circulation, 117*(15), 1973-1981. doi:10.1161/CIRCULATIONAHA.107.733212

Seyrantepe, V., Iannello, A., Liang, F., Kanshin, E., Jayanth, P., Samarani, S., . . . Pshezhetsky, A. V. (2010). Regulation of phagocytosis in macrophages by neuraminidase 1. *J Biol Chem, 285*(1), 206-215. doi:10.1074/jbc.M109.055475

Seyrantepe, V., Landry, K., Trudel, S., Hassan, J. A., Morales, C. R., & Pshezhetsky, A. V. (2004). Neu4, a novel human lysosomal lumen sialidase, confers normal phenotype to sialidosis and galactosialidosis cells. *J Biol Chem, 279*(35), 37021-37029. doi:10.1074/jbc.M404531200

Seyrantepe, V., Poupetova, H., Froissart, R., Zabot, M. T., Maire, I., & Pshezhetsky, A. V. (2003). Molecular pathology of NEU1 gene in sialidosis. *Hum Mutat, 22*(5), 343-352. doi:10.1002/humu.10268

Shelness, G. S., & Sellers, J. A. (2001). Very-low-density lipoprotein assembly and secretion. *Curr Opin Lipidol, 12*(2), 151-157.

Shen, M. M., Krauss, R. M., Lindgren, F. T., & Forte, T. M. (1981). Heterogeneity of serum low density lipoproteins in normal human subjects. *J Lipid Res, 22*(2), 236-244.

Shibuya, N., Goldstein, I. J., Broekaert, W. F., Nsimba-Lubaki, M., Peeters, B., & Peumans, W. J. (1987). The elderberry (Sambucus nigra L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. *J Biol Chem, 262*(4), 1596-1601.

Shiozaki, K., Yamaguchi, K., Takahashi, K., Moriya, S., & Miyagi, T. (2011a). Regulation of Sialyl Lewis Antigen Expression in Colon Cancer Cells by Sialidase NEU4. *Journal of Biological Chemistry, 286*(24), 21052-21061. doi:10.1074/jbc.M111.231191

Shiozaki, K., Yamaguchi, K., Takahashi, K., Moriya, S., & Miyagi, T. (2011b). Regulation of sialyl Lewis antigen expression in colon cancer cells by sialidase NEU4. *J Biol Chem, 286*(24), 21052-21061. doi:10.1074/jbc.M111.231191

Shireman, R. B., & Fisher, W. R. (1979). The absence of a role for the carbohydrate moiety in the binding of apolipoprotein B to the low density lipoprotein receptor. *Biochim Biophys Acta, 572*(3), 537-540.

Skalen, K., Gustafsson, M., Rydberg, E. K., Hulten, L. M., Wiklund, O., Innerarity, T. L., & Boren, J. (2002). Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. *Nature, 417*(6890), 750-754. doi:10.1038/nature00804

Smart, E. J., De Rose, R. A., & Farber, S. A. (2004). Annexin 2-caveolin 1 complex is a target of ezetimibe and regulates intestinal cholesterol transport. *Proc Natl Acad Sci U S A, 101*(10), 3450-3455. doi:10.1073/pnas.0400441101

Smutova, V., Albohy, A., Pan, X., Korchagina, E., Miyagi, T., Bovin, N., . . . Pshezhetsky, A. V. (2014). Structural basis for substrate specificity of mammalian neuraminidases. *PLoS One, 9*(9), e106320. doi:10.1371/journal.pone.0106320

Sobenin, I. A., Tertov, V. V., Orekhov, A. N., & Smirnov, V. N. (1991). Synergetic effect of desialylated and glycated low density lipoproteins on cholesterol accumulation in cultured smooth muscle intimal cells. *Atherosclerosis, 89*(2-3), 151-154.

Sparks, D. L., & Phillips, M. C. (1992). Quantitative measurement of lipoprotein surface charge by agarose gel electrophoresis. *J Lipid Res, 33*(1), 123-130.

Spitz, C., Winkels, H., Burger, C., Weber, C., Lutgens, E., Hansson, G. K., & Gerdes, N. (2016). Regulatory T cells in atherosclerosis: critical immune regulatory function and therapeutic potential. *Cell Mol Life Sci, 73*(5), 901-922. doi:10.1007/s00018-015-2080-2

Sprague, E. A., Moser, M., Edwards, E. H., & Schwartz, C. J. (1988). Stimulation of receptor-mediated low density lipoprotein endocytosis in neuraminidase-treated cultured bovine aortic endothelial cells. *J Cell Physiol, 137*(2), 251-262. doi:10.1002/jcp.1041370207

Stamatos, N. M., Liang, F., Nan, X., Landry, K., Cross, A. S., Wang, L. X., & Pshezhetsky, A. V. (2005). Differential expression of endogenous sialidases of human monocytes during cellular differentiation into macrophages. *FEBS J, 272*(10), 2545-2556. doi:10.1111/j.1742-4658.2005.04679.x

Stary, H. C., Chandler, A. B., Dinsmore, R. E., Fuster, V., Glagov, S., Insull, W., Jr., . . . Wissler, R. W. (1995). A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb Vasc Biol, 15*(9), 1512-1531.

Stary, H. C., Chandler, A. B., Glagov, S., Guyton, J. R., Insull, W., Jr., Rosenfeld, M. E., . . . Wissler, R. W. (1994). A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation, 89*(5), 2462-2478.

Steinberg, D. (1997). Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem, 272*(34), 20963-20966.

Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., & Witztum, J. L. (1989). Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med, 320*(14), 915-924. doi:10.1056/NEJM198904063201407

Steinberg, D., & Witztum, J. L. (2002). Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? *Circulation, 105*(17), 2107-2111.

Steinberg, D., & Witztum, J. L. (2009). Inhibition of PCSK9: a powerful weapon for achieving ideal LDL cholesterol levels. *Proc Natl Acad Sci U S A, 106*(24), 9546-9547. doi:10.1073/pnas.0904560106

Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L., & Steinberg, D. (1984). Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and

degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci U S A, 81*(12), 3883-3887.

Steinbrecher, U. P., Witztum, J. L., Parthasarathy, S., & Steinberg, D. (1987). Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. *Arteriosclerosis, 7*(2), 135-143.

Stocker, R., & Keaney, J. F., Jr. (2004). Role of oxidative modifications in atherosclerosis. *Physiol Rev, 84*(4), 1381-1478. doi:10.1152/physrev.00047.2003

Strecker, G., Peers, M. C., Michalski, J. C., Hondi-Assah, T., Fournet, B., Spik, G., . . . Durand, P. (1977). Structure of nine sialyl-oligosaccharides accumulated in urine of eleven patients with three different types of sialidosis. Mucolipidosis II and two new types of mucolipidosis. *Eur J Biochem, 75*(2), 391-403.

Sudano, I., Spieker, L. E., Hermann, F., Flammer, A., Corti, R., Noll, G., & Luscher, T. F. (2006). Protection of endothelial function: targets for nutritional and pharmacological interventions. *J Cardiovasc Pharmacol, 47 Suppl 2*, S136-150; discussion S172-136.

Superko, H. R., Pendyala, L., Williams, P. T., Momary, K. M., King, S. B., 3rd, & Garrett, B. C. (2012). High-density lipoprotein subclasses and their relationship to cardiovascular disease. *J Clin Lipidol, 6*(6), 496-523. doi:10.1016/j.jacl.2012.03.001

Tabas, I. (2002). Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J Clin Invest, 110*(7), 905-911. doi:10.1172/JCI16452

Tabas, I. (2010). Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol, 10*(1), 36-46. doi:10.1038/nri2675

Takashima, S. (2008). Characterization of mouse sialyltransferase genes: their evolution and diversity. *Biosci Biotechnol Biochem, 72*(5), 1155-1167.

Taniguchi, T., Ishikawa, Y., Tsunemitsu, M., & Fukuzaki, H. (1989). The structures of the asparagine-linked sugar chains of human apolipoprotein B-100. *Arch Biochem Biophys, 273*(1), 197-205.

Tardif, J. C., Gregoire, J., L'Allier, P. L., Ibrahim, R., Anderson, T. J., Reeves, F., . . . Investigators, C.-. (2008). Effects of the antioxidant succinobucol (AGI-1067) on human atherosclerosis in a randomized clinical trial. *Atherosclerosis, 197*(1), 480-486. doi:10.1016/j.atherosclerosis.2006.11.039

Tardif, J. C., McMurray, J. J., Klug, E., Small, R., Schumi, J., Choi, J., . . . Aggressive Reduction of Inflammation Stops Events Trial, I. (2008). Effects of succinobucol (AGI-1067) after an acute coronary syndrome: a randomised, double-blind, placebo-controlled trial. *Lancet, 371*(9626), 1761-1768. doi:10.1016/S0140-6736(08)60763-1

Tegos, T. J., Kalodiki, E., Sabetai, M. M., & Nicolaides, A. N. (2001). The genesis of atherosclerosis and risk factors: a review. *Angiology, 52*(2), 89-98.

Tertov, V. V., Orekhov, A. N., Sobenin, I. A., Gabbasov, Z. A., Popov, E. G., Yaroslavov, A. A., & Smirnov, V. N. (1992). Three types of naturally occurring modified lipoproteins induce intracellular lipid accumulation due to lipoprotein aggregation. *Circ Res, 71*(1), 218-228.

Tertov, V. V., Orekhov, A. N., Sobenin, I. A., Morrisett, J. D., Gotto, A. M., Jr., & Guevara, J. G., Jr. (1993). Carbohydrate composition of protein and lipid components in sialic acid-rich and -poor low density lipoproteins from subjects with and without coronary artery disease. *J Lipid Res, 34*(3), 365-375.

Thomson, M. J., Puntmann, V., & Kaski, J. C. (2007). Atherosclerosis and oxidant stress: the end of the road for antioxidant vitamin treatment? *Cardiovasc Drugs Ther, 21*(3), 195-210. doi:10.1007/s10557-007-6027-1

Tringali, C., Papini, N., Fusi, P., Croci, G., Borsani, G., Preti, A., . . . Monti, E. (2004). Properties of recombinant human cytosolic sialidase HsNEU2. The enzyme hydrolyzes monomerically dispersed GM1 ganglioside molecules. *J Biol Chem, 279*(5), 3169-3179. doi:10.1074/jbc.M308381200

Triplett, R. B., & Fisher, W. R. (1978). Proteolytic digestion in the elucidation of the structure of low density lipoprotein. *J Lipid Res, 19*(4), 478-488.

Trost, M., English, L., Lemieux, S., Courcelles, M., Desjardins, M., & Thibault, P. (2009). The phagosomal proteome in interferon-gamma-activated macrophages. *Immunity, 30*(1), 143-154. doi:10.1016/j.immuni.2008.11.006

Tupin, E., Nicoletti, A., Elhage, R., Rudling, M., Ljunggren, H. G., Hansson, G. K., & Berne, G. P. (2004). CD1d-dependent activation of NKT cells aggravates atherosclerosis. *J Exp Med, 199*(3), 417-422. doi:10.1084/jem.20030997

Tuzikov, A. B., Gambaryan, A. S., Juneja, L. R., & Bovin, N. V. (2000). Conversion of Complex Sialooligosaccharides into Polymeric Conjugates and their Anti-Influenza Virus Inhibitory Potency. *Journal of Carbohydrate Chemistry, 19*(9), 1191-1200. doi:10.1080/07328300008544143

Uslu, C., Taysi, S., Akcay, F., Sutbeyaz, M. Y., & Bakan, N. (2003). Serum free and bound sialic acid and alpha-1-acid glycoprotein in patients with laryngeal cancer. *Ann Clin Lab Sci, 33*(2), 156-159.

van Dijk, R. A., Kolodgie, F., Ravandi, A., Leibundgut, G., Hu, P. P., Prasad, A., . . . Tsimikas, S. (2012). Differential expression of oxidation-specific epitopes and apolipoprotein(a) in progressing and ruptured human coronary and carotid atherosclerotic lesions. *J Lipid Res, 53*(12), 2773-2790. doi:10.1194/jlr.P030890

van Pelt, J., Kamerling, J. P., Vliegenthart, J. F., Verheijen, F. W., & Galjaard, H. (1988). Isolation and structural characterization of sialic acid-containing storage material from mucolipidosis I (sialidosis) fibroblasts. *Biochim Biophys Acta, 965*(1), 36-45.

Varghese, J. N., McKimmbreschkin, J. L., Caldwell, J. B., Kortt, A. A., & Colman, P. M. (1992). THE STRUCTURE OF THE COMPLEX BETWEEN INFLUENZA-VIRUS NEURAMINIDASE AND SIALIC-ACID, THE VIRAL RECEPTOR. *Proteins-Structure Function and Genetics, 14*(3), 327-332.

Varki, A., & Angata, T. (2006). Siglecs--the major subfamily of I-type lectins. *Glycobiology, 16*(1), 1R-27R. doi:10.1093/glycob/cwj008

Varki, A., & Gagneux, P. (2012). Multifarious roles of sialic acids in immunity. *Ann N Y Acad Sci, 1253*, 16-36. doi:10.1111/j.1749-6632.2012.06517.x

Vatten, L. J., & Kvinnsland, S. (1990). Body height and risk of breast cancer. A prospective study of 23,831 Norwegian women. *Br J Cancer, 61*(6), 881-885.

Veniant, M. M., Pierotti, V., Newland, D., Cham, C. M., Sanan, D. A., Walzem, R. L., & Young, S. G. (1997). Susceptibility to atherosclerosis in mice expressing exclusively apolipoprotein B48 or apolipoprotein B100. *J Clin Invest, 100*(1), 180-188. doi:10.1172/JCI119511

Verbeek, R., Stoekenbroek, R. M., & Hovingh, G. K. (2015). PCSK9 inhibitors: Novel therapeutic agents for the treatment of hypercholesterolemia. *Eur J Pharmacol, 763*(Pt A), 38-47. doi:10.1016/j.ejphar.2015.03.099

Vinogradova, M. V., Michaud, L., Mezentsev, A. V., Lukong, K. E., El-Alfy, M., Morales, C. R., . . . Pshezhetsky, A. V. (1998). Molecular mechanism of lysosomal sialidase deficiency in galactosialidosis involves its rapid degradation. *Biochem J, 330 (Pt 2)*, 641-650.

Wada, T., Yoshikawa, Y., Tokuyama, S., Kuwabara, M., Akita, H., & Miyagi, T. (1999). Cloning, expression, and chromosomal mapping of a human ganglioside sialidase. *Biochem Biophys Res Commun, 261*(1), 21-27. doi:10.1006/bbrc.1999.0973

Wakabayashi, I., Sakamoto, K., Yoshimoto, S., & Kakishita, E. (1994). Serum sialic acid concentration and atherosclerotic risk factors. *J Atheroscler Thromb, 1*(2), 113-117.

Wang, D., Zaitsev, S., Taylor, G., d'Azzo, A., & Bonten, E. (2009). Protective protein/cathepsin A rescues N-glycosylation defects in neuraminidase-1. *Biochim Biophys Acta, 1790*(4), 275-282. doi:10.1016/j.bbagen.2009.01.006

Wang, J. C., & Bennett, M. (2012). Aging and atherosclerosis: mechanisms, functional consequences, and potential therapeutics for cellular senescence. *Circ Res, 111*(2), 245-259. doi:10.1161/CIRCRESAHA.111.261388

Wang, Y., Yamaguchi, K., Shimada, Y., Zhao, X., & Miyagi, T. (2001). Site-directed mutagenesis of human membrane-associated ganglioside sialidase: identification of amino-acid residues contributing to substrate specificity. *Eur J Biochem, 268*(8), 2201-2208.

Wang, Y., Yamaguchi, K., Wada, T., Hata, K., Zhao, X., Fujimoto, T., & Miyagi, T. (2002). A close association of the ganglioside-specific sialidase Neu3 with caveolin in membrane microdomains. *J Biol Chem, 277*(29), 26252-26259. doi:10.1074/jbc.M110515200

Weber, C., Zernecke, A., & Libby, P. (2008). The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol, 8*(10), 802-815. doi:10.1038/nri2415

Wernette-Hammond, M. E., Lauer, S. J., Corsini, A., Walker, D., Taylor, J. M., & Rall, S. C., Jr. (1989). Glycosylation of human apolipoprotein E. The carbohydrate attachment site is threonine 194. *J Biol Chem, 264*(15), 9094-9101.

Whitman, S. C. (2004). A practical approach to using mice in atherosclerosis research. *Clin Biochem Rev, 25*(1), 81-93.

Williams, K. J., Petrie, K. A., Brocia, R. W., & Swenson, T. L. (1991). Lipoprotein lipase modulates net secretory output of apolipoprotein B in vitro. A possible pathophysiologic explanation for familial combined hyperlipidemia. *J Clin Invest, 88*(4), 1300-1306. doi:10.1172/JCI115434

Williams, K. J., & Tabas, I. (1998). The response-to-retention hypothesis of atherogenesis reinforced. *Curr Opin Lipidol, 9*(5), 471-474.

Willnow, T. E. (1997). Mechanisms of hepatic chylomicron remnant clearance. *Diabet Med, 14 Suppl 3*, S75-80. doi:10.1002/(SICI)1096-9136(199708)14:3+<S75::AID-DIA449>3.0.CO;2-9

Winter, R. M., Swallow, D. M., Baraitser, M., & Purkiss, P. (1980). Sialidosis type 2 (acid neuraminidase deficiency): clinical and biochemical features of a further case. *Clin Genet, 18*(3), 203-210.

World Health Organization, W. (2017). http://www.who.int/cardiovascular_diseases/en/

Yamaguchi, K., Hata, K., Koseki, K., Shiozaki, K., Akita, H., Wada, T., . . . Miyagi, T. (2005). Evidence for mitochondrial localization of a novel human sialidase (NEU4). *Biochem J, 390*(Pt 1), 85-93. doi:10.1042/BJ20050017

Yamaguchi, K., Hata, K., Wada, T., Moriya, S., & Miyagi, T. (2006). Epidermal growth factor-induced mobilization of a ganglioside-specific sialidase (NEU3) to membrane ruffles. *Biochem Biophys Res Commun, 346*(2), 484-490. doi:10.1016/j.bbrc.2006.05.136

Yamaguchi, K., Shiozaki, K., Moriya, S., Koseki, K., Wada, T., Tateno, H., . . . Miyagi, T. (2012). Reduced susceptibility to colitis-associated colon carcinogenesis in mice lacking plasma membrane-associated sialidase. *PLoS One, 7*(7), e41132. doi:10.1371/journal.pone.0041132

Yamamoto, N., & Kumashiro, R. (1993). Conversion of vitamin D3 binding protein (group-specific component) to a macrophage activating factor by the stepwise action of beta-galactosidase of B cells and sialidase of T cells. *J Immunol, 151*(5), 2794-2802.

Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L., & Russell, D. W. (1984). The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell, 39*(1), 27-38.

Yamamoto, T., Lu, C., & Ryan, R. O. (2011). A two-step binding model of PCSK9 interaction with the low density lipoprotein receptor. *J Biol Chem, 286*(7), 5464-5470. doi:10.1074/jbc.M110.199042

Yang, C. Y., Chen, S. H., Gianturco, S. H., Bradley, W. A., Sparrow, J. T., Tanimura, M., . . . et al. (1986). Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. *Nature, 323*(6090), 738-742. doi:10.1038/323738a0

Yang, C. Y., Kim, T. W., Weng, S. A., Lee, B. R., Yang, M. L., & Gotto, A. M., Jr. (1990). Isolation and characterization of sulfhydryl and disulfide peptides of human apolipoprotein B-100. *Proc Natl Acad Sci U S A, 87*(14), 5523-5527.

Ye, Q., Chen, Y., Lei, H., Liu, Q., Moorhead, J. F., Varghese, Z., & Ruan, X. Z. (2009). Inflammatory stress increases unmodified LDL uptake via LDL receptor: an alternative pathway for macrophage foam-cell formation. *Inflamm Res, 58*(11), 809-818. doi:10.1007/s00011-009-0052-4

Yla-Herttuala, S., Solakivi, T., Hirvonen, J., Laaksonen, H., Mottonen, M., Pesonen, E., . . . Nikkari, T. (1987). Glycosaminoglycans and apolipoproteins B and A-I in human aortas. Chemical and immunological analysis of lesion-free aortas from children and adults. *Arteriosclerosis, 7*(4), 333-340.

Yogalingam, G., Bonten, E. J., van de Vlekkert, D., Hu, H., Moshiach, S., Connell, S. A., & d'Azzo, A. (2008). Neuraminidase 1 is a negative regulator of lysosomal exocytosis. *Dev Cell, 15*(1), 74-86. doi:10.1016/j.devcel.2008.05.005

Yoshino, H., Miyashita, K., Miyatani, N., Ariga, T., Hashimoto, Y., Tsuji, S., . . . et al. (1990). Abnormal glycosphingolipid metabolism in the nervous system of galactosialidosis. *J Neurol Sci, 97*(1), 53-65.

Yu, C., Chen, Y., Cline, G. W., Zhang, D., Zong, H., Wang, Y., . . . Shulman, G. I. (2002). Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem, 277*(52), 50230-50236. doi:10.1074/jbc.M200958200

Yu, X. H., Fu, Y. C., Zhang, D. W., Yin, K., & Tang, C. K. (2013). Foam cells in atherosclerosis. *Clin Chim Acta, 424*, 245-252. doi:10.1016/j.cca.2013.06.006

Zanchetti, G., Colombi, P., Manzoni, M., Anastasia, L., Caimi, L., Borsani, G., . . . Bresciani, R. (2007). Sialidase NEU3 is a peripheral membrane protein localized on the cell surface and in endosomal structures. *Biochem J, 408*(2), 211-219. doi:10.1042/BJ20070503

Zanoteli, E., van de Vlekkert, D., Bonten, E. J., Hu, H., Mann, L., Gomero, E. M., . . . d'Azzo, A. (2010). Muscle degeneration in neuraminidase 1-deficient mice results from infiltration of the muscle fibers by expanded connective tissue. *Biochim Biophys Acta, 1802*(7-8), 659-672. doi:10.1016/j.bbadis.2010.04.002

Zarins, C. K., Giddens, D. P., Bharadvaj, B. K., Sottiurai, V. S., Mabon, R. F., & Glagov, S. (1983). Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress. *Circ Res, 53*(4), 502-514.

Zeiher, A. M., Fisslthaler, B., Schray-Utz, B., & Busse, R. (1995). Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells. *Circ Res, 76*(6), 980-986.

Zhang, S. H., Reddick, R. L., Piedrahita, J. A., & Maeda, N. (1992). Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science, 258*(5081), 468-471.

Zhang, Y., Albohy, A., Zou, Y., Smutova, V., Pshezhetsky, A. V., & Cairo, C. W. (2013). Identification of selective inhibitors for human neuraminidase isoenzymes using C4,C7-modified 2-deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA) analogues. *J Med Chem, 56*(7), 2948-2958. doi:10.1021/jm301892f

Zhou, Q., & Liao, J. K. (2009). Rho kinase: an important mediator of atherosclerosis and vascular disease. *Curr Pharm Des, 15*(27), 3108-3115.

Zhou, X. Y., Morreau, H., Rottier, R., Davis, D., Bonten, E., Gillemans, N., . . . d'Azzo, A. (1995). Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with overexpressing erythroid precursor cells. *Genes Dev, 9*(21), 2623-2634.

Zoungas, S., & Patel, A. (2010). Cardiovascular outcomes in type 2 diabetes: the impact of preventative therapies. *Ann N Y Acad Sci, 1212*, 29-40. doi:10.1111/j.1749-6632.2010.05837.x